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# MARINE INVASIVE SPECIES: FLUORESCENCE APPLICATIONS FOR BALLAST WATER REGULATORY COMPLIANCE AND USE OF FRESHWATER AS A BIOSECURITY TOOL FOR BIOFOULING

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University of Plymouth

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**UNIVERSITY OF  
PLYMOUTH**

**MARINE INVASIVE SPECIES: FLUORESCENCE APPLICATIONS  
FOR BALLAST WATER REGULATORY COMPLIANCE AND USE  
OF FRESHWATER AS A BIOSECURITY TOOL FOR BIOFOULING**

By

**MARIA CECILIA TRINDADE DE CASTRO**

A thesis submitted to the University of Plymouth  
in partial fulfilment for the degree of

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School of Biological and Marine Sciences

[In collaboration with  
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**UNIVERSITY OF PLYMOUTH**  
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*"(...) See the line where the sky meets the sea?  
It calls me  
No one knows  
How far it goes  
If the wind in my sail on the sea  
Stays behind me  
One day I'll know  
If I go there's just no telling how far I'll go (...)"*

Lin-Manuel Miranda

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---

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## Author's Declaration

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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# Abstract

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## Marine Invasive Species: Fluorescence applications for ballast water regulatory compliance and use of freshwater as a biosecurity tool for biofouling

Maria Cecilia Trindade de Castro

The present thesis addresses the role of shipping as an unintentional and very efficient pathway for spreading aquatic non-native species around the globe, through two major vectors: ballast water and biofouling. The challenge with ballast water is that a myriad of organisms are transported across natural barriers before dispersal, transcending biogeographic regions and this wholesale movement of marine life contributes to the spread of diseases and to the homogenization of coastal habitats. The United Nations nominated the issue as one of the four greatest threats to the oceans, causing extremely severe environmental, economic and public health impacts.

This research is primarily focussed on the investigation of portable instruments (fluorometers) developed to support inspections regimes on the efficiency of ballast water management systems required by international regulations and compare them to well-established research tools, e.g. flow cytometry. Viability tests of phytoplankton groups in the size range defined by these regulations in natural assemblies were conducted as well as in treated ballast water samples. An overall good correlation between the measurements taken with the fluorometers and in comparison with the flow cytometry analysis was found. Analysis of treated ballast water samples showed a large variation in the number of viable cells, however indicating the same risk on all occasions for regulatory purposes. In addition, experiments to examine the application of flow cytometry and fluorometry in characterizing natural phytoplankton communities, with special attention to cell size, found relevant results in the context of the size class distribution based on flow cytometry and semi-quantification using chlorophyll as a proxy for cell density. Found results may indicate the need for further refinement of portable fluorometers with filtration steps and in use for compliance issues.

Species can also be transported on virtually all of the submerged areas on ships and so antifouling systems are used to reduce fouling. However, with increased regulation of biocides used in antifouling coatings, there is a need to find efficient and sustainable alternatives. In this regard, experiments using low salinity to kill fouling organisms in areas of the ship where it is difficult to coat and therefore tend to accumulate fouling organisms, e.g. ships sea-chest, were conducted. Results showed high levels of mortality in mature biofouling communities exposed to two hour treatment with a salinity of 7 psu. Low salinity treatments can offer an environmentally friendly biosecurity tool for minimising and controlling ships sea-chest biofouling that is simple and would not cause undue delay or costs for the ship.

Large quantities of non-native species are transported daily either through ballast water or ships biofouling; however, the rate of establishment of invasive species is unclear and associated to the interplay of varied factors. Moreover, in many cases, it is difficult to disentangle the level of influence of the different vectors and pathways in this transfer. As a result, a general approach to control and minimise the unintentional transfer of non-native species through shipping should consider the adoption of integrated studies where important vectors are considered together as the best way to move forward. In the present case, it means the necessity of having biofouling under control together with ballast water.

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## List of Abbreviations

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AFS – Anti-fouling Systems

BWMS – Ballast Water Management System

BWTS - Ballast Water Treatment System

BWM Convention – International Convention for the Control and Management of Ships Ballast Water and Sediments / Ballast Water Management Convention

CFU - Colony Forming Unit

GEF – Global Environment Fund

GloBallast – Global Ballast Water Programme

HELCOM - Baltic Marine Environment Protection Commission

Helsinki Convention - Convention on the Protection of the Marine Environment of the Baltic Sea Area

IMO – International Maritime Organization

MEPC – Marine Environment Protection Committee

MMA – Brazilian Ministry of Environment

NORMAM 20 – Brazilian Maritime Authority Standard for the Management of Ships Ballast Water

OSPAR Convention – Convention for the Protection of the Marine Environment of the North-East Atlantic

RMT – Review of Maritime Transport

TBT – Tributyltin

UN – United Nations

UNCTAD – United Nations Conference of Trade and Development

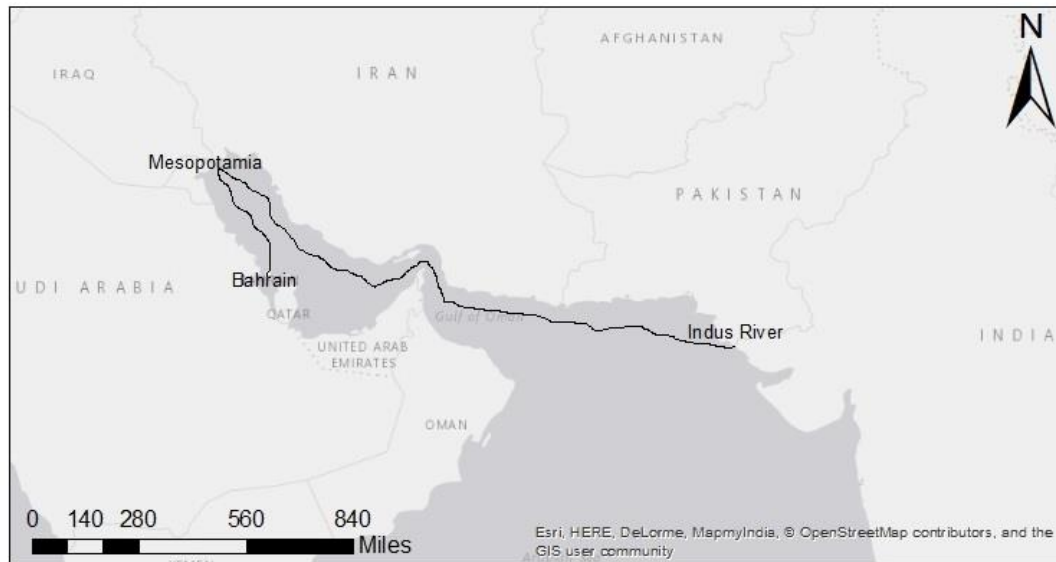
UNDP – United Nations Development Programme

## **Chapter 1 : Shipping as a pathway for the transfer and spread of non-native species**

## 1.1. General aspects of shipping

Sea transport has played a very important role since the early stages of economic development. First known sea trade dates back 5,000 years between Mesopotamia, Bahrain and the Indus River in western India (Stopford, 2009) (Figure 1.1). There were long routes, busy ports and intense and diverse trade around the Indian Ocean during the 11<sup>th</sup> to 15<sup>th</sup> century throughout a European dominance from the 14<sup>th</sup> Century until recently (Lucassen & Unger, 2011; Paine, 2014; Ojala & Tenold, 2017).

The shipping industry is omnipresent in human history and its dominance was intrinsically related to dominant powerful nations or empires e.g. Mesopotamia, Phoenicia, Roman Empire, British Empire and United States, among others. In a globalized world however the shipping industry tends to be more tied to the capacity of trade / business of big companies than to nations.



**Figure 1.1. First known sea trade network in the human history (Mesopotamia, Bahrain and Indus River – western India), adapted from Stopford, 2009.**

Navigation of the Atlantic Ocean during the 15th Century lead by European Countries, laid the foundation for a global sea trade network that would dominate shipping for the next



500 years (Stopford, 2009). During the second half of the 19<sup>th</sup> century the world witnessed the shift from sail to steam and from wood to steel brought by the Europeans which consolidated their hegemony in the period; meanwhile the specialisation of ships and the use of diesel marked the 20<sup>th</sup> Century (Ojala & Tenold, 2017).

According to the 2017 United Nations Conference Trade and Development Report, China, United States of America and Germany together were responsible for 30% of global exports in 2016 (Figure 1.2). However, the world export value in 2016 was down from 2008 (US\$16.1 trillion), when the last global financial crisis hit. Within the developed world, a merchandise trade deficit was recorded while for developing economies and economies in transition a surplus was recorded but lower than three years before (UNCTAD/RMT/2017). Developing countries account for the largest share of global seaborne trade (60% of all goods loaded and 56% of all goods unloaded), while developed economies numbers corresponded to 34% and 43% respectively. Transition economies accounted for 6% of goods loaded and only 1% of global goods unloaded (MKC/IMO, 2012).

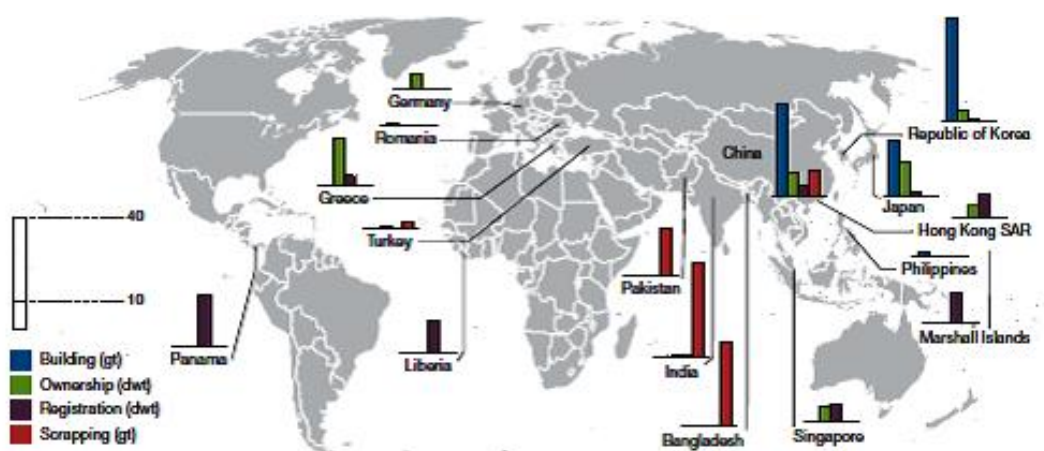


**Figure 1.2. Major world import flows in 2016 in billions of US dollars (UNCTAD/RMT/2017).**

The top five ship owning countries are Greece, Japan, China, Germany and Singapore, corresponding to nearly 50% of the world dead weight tonnage (dwt) (Figure 1.3). From

Latin America only Brazil is in the top 35 ship owning countries (UNCTAD/RMT/2017). With regards to the Flag registries, the top five countries are Panama, Liberia, Marshall Islands, China Hong Kong SAR and Singapore (57.8%) (Figure 1.3).

Another aspect intrinsically related to the shipping industry refers to the construction and dismantling of ships. Three countries (Republic of Korea, China and Japan) were responsible for > 90% of world tonnage and four for ca. 95% of ship scrapping (India, Bangladesh, Pakistan and China) in 2016 (Figure 1.3) (UNCTAD/RMT/2017).



**Figure 1.3. Main building, ownership, Flag registries and scrapping countries in the world in 2016 (UNCTAD/RMT/ 2017).**

In 2011 the UNCTAD reported the world fleet of propelled sea-going merchant ships equal or more than 100 gross tonnage (GT) comprised 104,304 ships with an average age of 22 years; within that, the cargo carrying fleet accounted for more than 55,000 ships with 991,173,697 gross tonnage and with an average age of 19 years (UNCTAD/RMT/2011). In 2017, the world fleet reached 1.9 billion dead weight tonnage, from which bulk carriers explained 43 % of the fleet, followed by oil tankers (29 %) and container ships (13 %) while other types correspond to 11.3% and general cargo ships to 4% (UNCTAD/RMT/2017).

The facts and figures associated with the shipping industry provide the necessary background to set out its importance in the world economy. And, from an ecological perspective, provide an idea of the threat represented by shipping as a very efficient pathway for transferring and spreading non-native species through two major vectors: ballast water and biofouling.

## **1.2. Marine non-native species**

Non-native species can range from viruses and bacteria to fungi, protists, plants, and animals and its introduction and spread is acknowledged as a major threat to the biodiversity of the world (Keller et al., 2011; Gurevitch & Padilla, 2014). However, it is important to highlight that not every introduced species will become an invasive or harmful species causing impacts over an individual, a population or towards an entire community. Species described outside its natural range are considered non-native or introduced species; when capable of establishing a population in the new environment, they become established or naturalised (Richardson et al., 2000). When its presence interferes in the survivability of other native species or causes damage to the environment, human health, property and resources, then this species is called invasive or harmful (Hilliard et al., 1997; Elliot, 2003). Nevertheless, species not necessarily behave the same way in a new environment which means that the same species can become an invasive species in one area whereas keeping the status of non-native or established in another area. In addition to species physiological traits, biotic and abiotic characteristics of the new environment, the propagule pressure, composed by the propagule size, or the number of individuals in the propagule, and the propagule number, which means the rate of arrival, has a fundamental role in the invasion process (Simberloff, 2009).

Non-native, non-indigenous, exotic, introduced or alien species are species originally not described / not recorded for a given geographic area. These species can be intentionally

(e.g. for aquaculture purposes) or unintentionally (e.g. ballast water) introduced. Biological invasions comprise both natural range expansions (e.g. due to sea surface warming), as well as human-mediated ones (Carlton, 1985). Allied to the increasing trade around the world, the advent of new shipping routes, climate change, over-exploited seafood stocks, physical alteration of marine habitats and biological invasions themselves, boost the opportunities for new invasions in the aquatic environment (Williams et al., 2013; Ware et al., 2014; Hall-Spencer & Allan, 2015; Castro et al., 2017). Following the introduction of a new species, a series of factors will determine the success of the species in the new environment, environmental similarity for instance might increase the odds of a successful invasion (Keller et al., 2011); other important parameters are the propagule pressure, or the “introduction effort”, interspecies competition, species-specific tolerances to abiotic factors and transportation stress (Lockwood et al., 2005; Lockwood & Somero, 2011; Briski et al, 2012).

There are a few conditions that should be addressed when assessing whether or not a non-native species should be also considered harmful or invasive. The displacement of native species by non-native species via competition; non-native species predation on native ones causing a decrease on native species biomass; parasitism or illness caused by non-native species; and when economic losses are associated to the introduction of non-native species (e.g. encrustation in water pipes and cooling systems, hull fouling) Hilliard et al. (1997).

Parker et al. (1999) nominated five stages to be observed when assessing the impact of a non-native species in a new ecosystem; effects over native species (e.g. mortality); hybridisation; interference at community level (e.g. species richness) and on the population dynamics and finally on how the presence of non-native species disturbs ecosystem processes (e.g. primary productivity). Nevertheless, some of the impacts previously mentioned are hard to observe or quantify and are usually perceived when a

later stage of invasion is already in course. Invasive species are well known for their negative impacts over ecosystems and also for economic and social implications associated. Pimentel et al. (2005) estimated over 120 billion U.S. dollars of economic losses annually associated to invasive species in the Great Lakes only in the United States.

Environmental impacts arising from biological invasions over phytoplankton populations can, for instance, directly or indirectly cause shifts in phytoplankton community composition and biomass and affect food webs as well as the carbon cycle (Bastviken et al., 1998).

Three phases are usually described to illustrate the relationship between shipping and the rate of non-native aquatic invasions. A first phase before the industrial revolution where no direct relationship was seen; after 1800 when the transfer of non-native species started being facilitated by the construction of canals and other cargo outflow routes to drain the production, period that coincides with the advent of the steam propulsion; and the current phase, as “the era of globalization” of biological invasions (Lodge et al. 2006; Findley & O’Rourke 2007; Hulme, 2009).

A key aspect remains on the fact that after being introduced it is very difficult to eradicate a species, which means that the most effective way of dealing with non-native species is by adopting a proactive approach: avoiding their introduction or eradicating them in an early stage of invasion (Lodge et al., 2009).

### **1.3. Marine non-native species through shipping: Ballast Water**

As defined in the International Convention for the Control and Management of Ships’ Ballast Water and Sediments, 2004, ballast water means water with its suspended matter taken on board a ship to control trim (the distribution of the load in a ship), draught,

stability or stresses of the ship. Commercial ships need to maintain a constant balance of the loads carried by them, therefore when loading / unloading cargo on a port, they generally need to unload / load water in their ballast tanks respectively. The density and diversity of invasive species are usually higher in areas where shipping is extensive in ports around the world (Clark et al., 2015). Ships ballast tanks can carry between 3,000 to 7,000 species per ship; consequently the water and the organisms contained within it are able to easily cross natural barriers and biogeographic regions before being discharged in a new coastal area (Carlton & Geller, 1993; Gollasch, 1996; Gollasch et al., 2002, Endrensen et al., 2004). This movement represents a significant contribution towards the homogenisation of coastal habitats, can affect the ecological balance of aquatic ecosystems and can promote the spread of diseases, among other possible impacts (Ruiz et al., 2000; Drake & Lodge, 2004; Rahel, 2007, Katsanevakis et al., 2014). Phytoplankton species are the best candidates to survive among all the species in ballast tanks considering they are small, capable of forming cysts and deal with prolonged periods of darkness (McCarthy and Crowder, 2000). A study conducted in Australia found diatom and dinoflagellate resting spores in all ships carrying considerable amounts of sediments, including cysts of the toxic dinoflagellates *Alexandrium catenella*, *Alexandrium tamarense* and *Gymnodinium catenatum* (Hallegraeff & Bolch, 1992).

The transfer and spread of non-native species and / or pathogens, disease-causing microorganisms, through ballast water differ from other ship-related sources of pollution (e.g. oil or chemical spillage) due to the fact of resulting from an activity intrinsically related to the operation of ships (Leal-Neto, 2007). Ship-mediated introduction of marine non-native species date back to 1600s with the detection of the green macroalgae *Halimeda opuntia* in the Caribbean, originally from the Pacific Ocean (Kooistra & Verbruggen, 2005; Carlton & Ruiz, 2015). Scientific records however are from the beginning of the 20<sup>th</sup>

century after a mass occurrence of *Odontella sinensis* in the North Sea, a diatom originally native to the Pacific Ocean and now considered a cosmopolitan species (Ostenfeld, 1908).

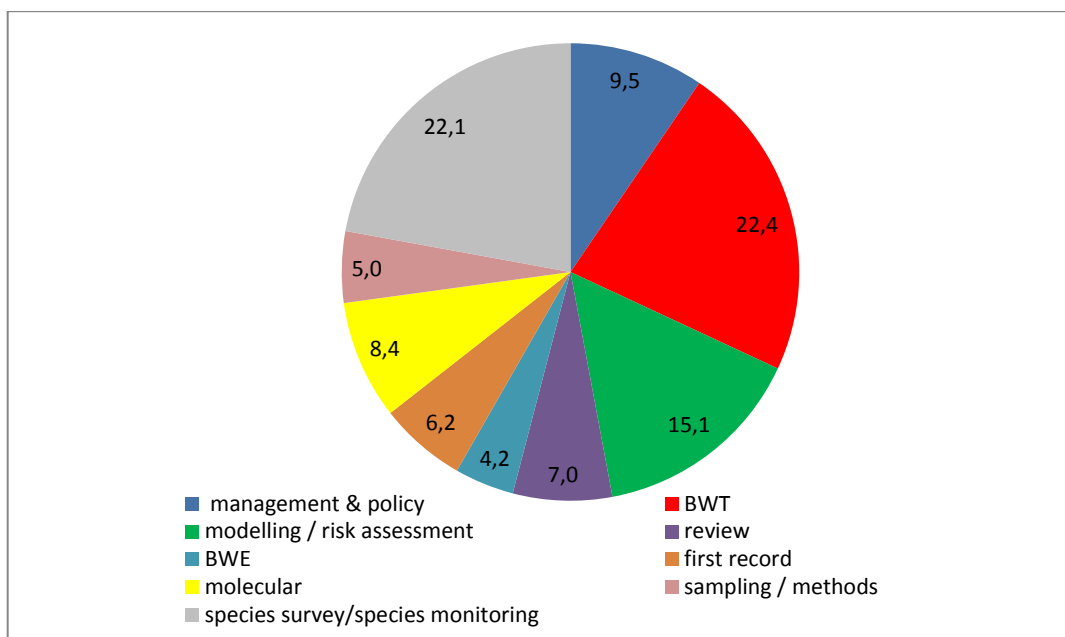
Records on the occurrence of non-native invasive species around the globe have got more common possibly considering the efforts in terms of research and international initiatives in the last thirty years. Some invasive species have been widely reported and must be taken as critical examples of the threat represented by the introduction of species outside their natural range. Zebra Mussel (*Dreissena polymorpha*) is a small freshwater mussel from Eastern Europe (Black Sea) and was first recorded as introduced into the Great Lakes in the mid-1980s (Hebert et al. 1989). This bivalve accounts for one of at least 57 non-native species that have become established in the region (Rothlisberger et al., 2012). After more than thirty years of its first record, this single species has become one of the most widespread and abundant; able to alter food webs and the biogeochemistry of the Great Lakes (Strayer, 2009). *Mnemiopsis leidyi*, known as comb jelly or sea walnut, is native to the Atlantic coast of North and South America and was accidentally introduced by ballast water in the Black Sea during the 1980s (Kideys, 1994). This one species combined with overfishing and eutrophication brought severe ecological and economic consequences to the region (Leppäkoski & Mihnea, 1996). Many other non-native species were probably introduced in Europe from ballast water discharges and sediments from ballast tanks, e.g. *Eriocheir sinensis*, *Amphibalanus improvisus* (Katsanevakis et al., 2014).

The International Maritime Organization and the World Health Organization recognise ballast water discharges as potentially harmful. According to an assessment of the United States Office of Technology from early 1990s, approximately 15% of non-native species introduced in the United States are known to cause disorders of major ecological and / or economic impacts. Ballast water discharges can also act as vectors for spreading pathogens and therefore increase the risk of epidemic diseases around the Globe (Ruiz et

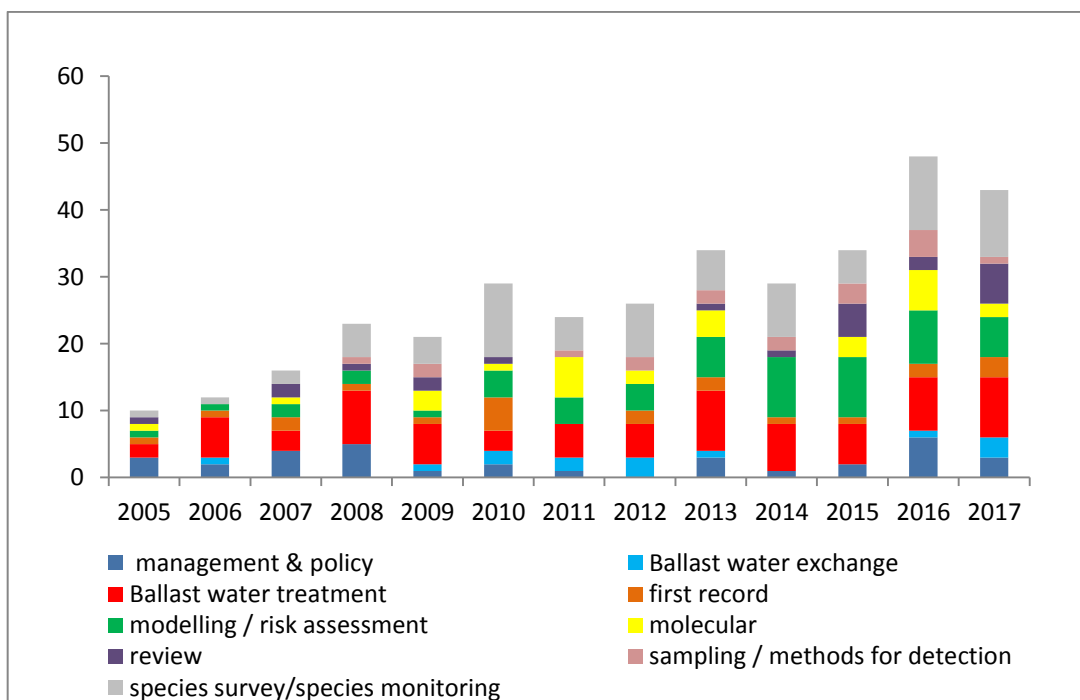
al. 2000). Estimated volumes of 3 to 10 billions of tonnes of ballast water per year can be carried by commercial ships which can explain the potential of ships ballast water as a facilitator of biological invasions (Carlton, 1989; Carlton et al., 1995). Bulk vessels known as Valemax, for instance, are mineral vessels with capacity for 400,000 tonnes of ore and carry estimated volumes of 10,000 to 120,000 m<sup>3</sup> of ballast water per journey (Pereira, 2012).

A literature review considering the period between 2005 and 2017 in the Thomson Reuters Web of Science <sup>TM</sup> database using the expressions “ballast water” and “invasive species” for searching, showed almost 400 papers. From this total, 349 papers were selected and classified according to their main subjects of research (categories): (1) management & policy, (2) ballast water exchange, (3) ballast water treatment, (4) modelling / risk assessment, (5) molecular studies, (6) new records, (7) review, (8) sampling / methodologies for detection and (9) invasive species surveys / monitoring. 22 % of papers focussed on ballast water treatment followed by studies on the survey and monitoring of species (Figure 1.4). A relatively constant increase in the number of papers can be observed over the time for each topic (Figure 1.5). Results might suggest a joint effort of science and the shipping industry over the last decade as recognition of the threat represented by invasive species through ballast water and on the necessity of taking action. Furthermore, an increased number of studies where modelling and risk assessment were primarily addressed is observed along the years, possibly denoting the adoption of a more proactive approach. According to Bailey (2015) the explosion of research focused on ballast water had an inflection point with the review published by Carlton (1985) with about 400 papers published hitherto.





**Figure 1.4. Percentage of papers searched on Web of Science database using the expressions “ballast water” and “invasive species” according to the main subject of research for the period 2005 - 2017.**

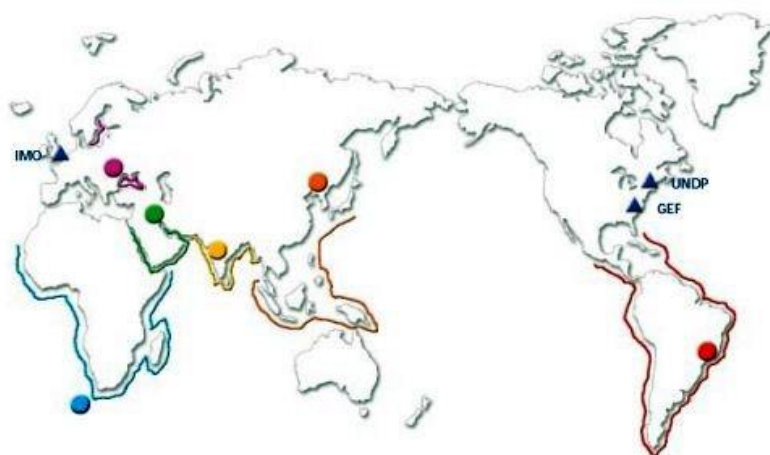


**Figure 1.5. Number of papers per main subject of research per year for the period 2005 – 2017.**

### 1.3.1. Regulations on ballast water

With regards to international initiatives for controlling / minimizing the spread of invasive species through ballast water, first International Maritime Organization guidelines were launched in 1991 by the Marine Environment Protection Committee: International Guidelines for preventing the introduction of unwanted aquatic organisms and pathogens from ships ballast water and sediment discharges (resolution MEPC.50(31)). In the next year, during the United Nations Conference on Environment and Development, held in Rio de Janeiro, the Convention of Biological Diversity was adopted. The Article 8 (h) of the Convention of Biological Diversity has brought into attention the need of addressing and minimizing the problem represented by invasive species around the Globe. The United Nations, through the International Maritime Organization, its specialised agency for safety, security and environmental performance of international shipping, nominated the issue as one of the four greatest threats to the world's oceans, in view of the negative impacts it can cause for the environment, the economy and human health. In that sense and under the scope of the International Maritime Organization, a major programme on the vector ballast water was launched in 2000, called "*Removal of Barriers to the Effective Implementation of Ballast Water Control and Management Measures in Developing Countries*" or simply Global Ballast Water Programme (GloBallast). The Programme was a joint initiative from the Global Environment Facility as funding agency, United Nations Development Programme as implementing agency and the International Maritime Organization as the executing agency. The GloBallast had two phases, the first one (2000-2004) had the primary goal of preparing developing countries to the adoption of an International Convention on ballast water, as well as to support them on the implementation of voluntary guidelines provided in the Assembly Resolution A.868(20) "guidelines for the control and management of ships ballast water to minimize the transfer of harmful aquatic organisms and pathogens", adopted on 27 November 1997. During the

first phase six ports were chosen to represent major developing regions in the world: Sepetiba (latter renamed as Itaguaí) (Brazil), Dalian (China), Mumbai (India), Khark island (Iran), Odessa (Ukraine) and Saldanha (South Africa), where risk assessment analyses were developed (Figure 1.6). A second phase called “GloBallast Partnerships” was launched in 2007 coming to an end ten years later. The second phase had the main objective of multiplying the results obtained in the original project, focussing on national policies, legal and institutional reforms in developing countries not addressed in the first phase. Sub-regions included in the second phase were: Caribbean, Mediterranean, Red Sea and Gulf of Aden, the South East Pacific and the West Coast of Africa and the South Pacific.



Demonstration Site	Pilot Country	Region
 Dalian	China	Asia / Pacific
 Khark Is	I.R. Iran	ROPME Sea Area & Red Sea
 Mumbai	India	South Asia
 Odessa	Ukraine	Eastern Europe
 Saldanha	South Africa	Africa
 Sepetiba / Itaguaí	Brazil	South America

**Figure 1.6. GloBallast Phase 1: Demonstration sites and Pilot Countries according to their regions.** (Source: <http://archive.iwlearn.net/globallast.imo.org/the-globallast-pilot-phase-2000-2004/index.html>, accessed in 20/02/2018).

Apart from the International Ballast Water Convention adopted in 2004 that came into force in September, 2017, regional agreements were also adopted to tackle the problem prior to the entered into force of the BWMC. The Barcelona Convention for the Protection of the Mediterranean Sea against Pollution includes invasive species monitoring as a priority subject and has an action plan concerning invasive species in the Mediterranean Sea. Likewise the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention), and the Convention on the Protection of the Marine Environment of the Baltic Sea Area (Helsinki Convention) adopted in 2008 joint guidelines on the risks of introduction and spread of non-native species in the North-East Atlantic and in the Baltic Sea (Castro et al., 2017). In addition a few unilateral regulations entered into force before the International Maritime Organization Convention (e.g. Brazilian Maritime Authority Standard for the Management of Ships Ballast Water (NORMAM-20), 2005; Canada Shipping Act 2001, 2011; Standards for Living Organisms in Ships Ballast Water Discharged in U.S. Waters, 2012).

#### **1.4. Marine non-native species through shipping: Biofouling**

A ship can provide many possibilities for the transportation of organisms: ballast water, hulls, propellers, sea-chests among other areas taking into account organisms can travel in different life stages and forms (free-living and fouled). Biofouling is considered a highly efficient vector for the transference of non-native species around the world together with ballast water. Species can be transported in virtually all submerged areas of ships and one common way of preventing / minimizing the fouling of invertebrates is the use of anti-fouling systems (AFS) in paints for coating the ships.

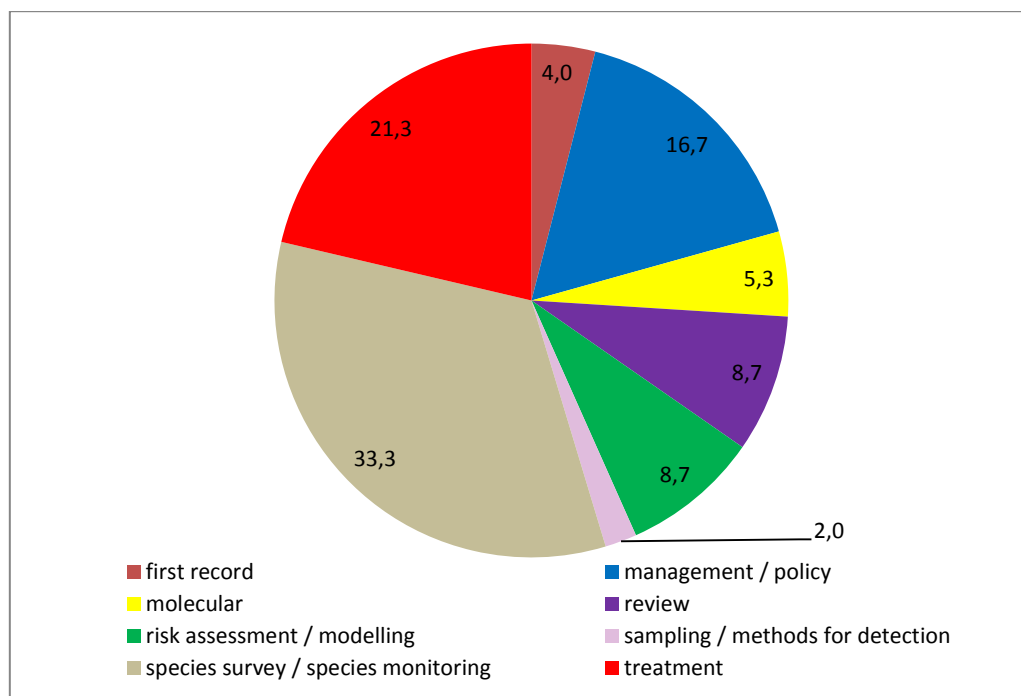
Biofouling results from the colonization process of bacteria, algae and or sessile invertebrates over submerged natural or man-made surfaces. Fouling organisms themselves can act as substrata for other organisms as well. Despite being a natural

process it is usually considered a problem in man-made structures, like ship hulls and hydropower plants with ecologic and economic costs associated.

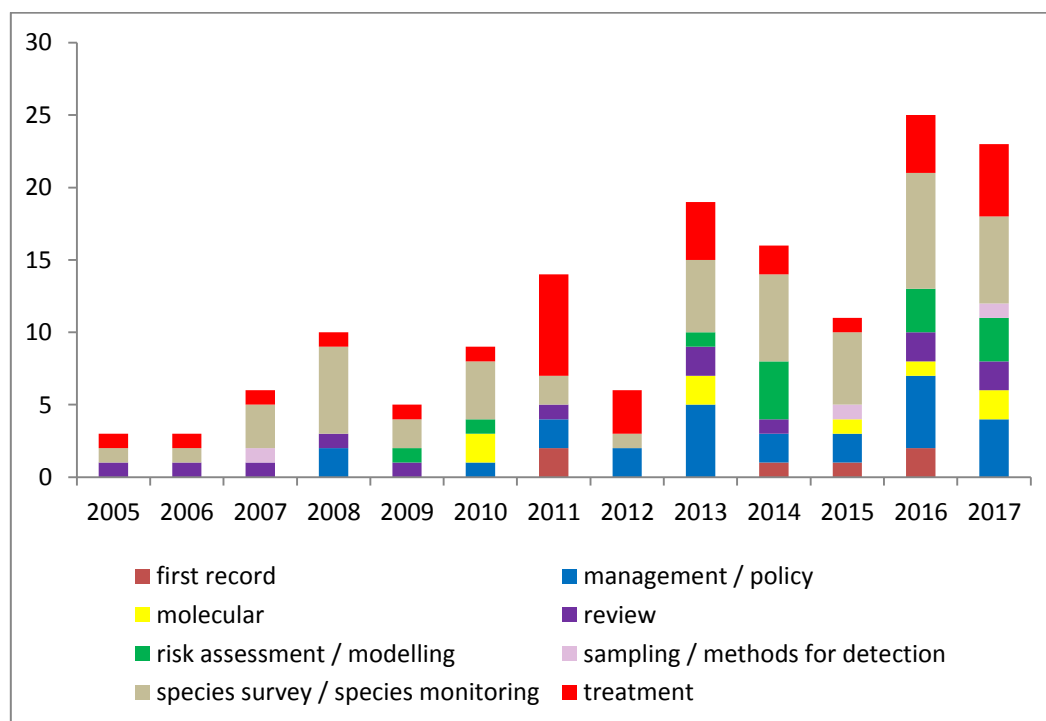
Usual practices to avoid biofouling on ships are related to the use of biocides that normally contain chemicals to kill or prevent the adherence of fouling organisms. Copper is inefficient after short periods and was replaced by organotin compounds (e.g. tributyltin - TBT) since the Second World War until 2008 when the International Maritime Organization imposed a ban on the use of TBT in Anti-Fouling Systems (Da Gama et al., 2009).

Areas of ships difficult to access and coat are called niche areas and represent higher risk areas for biofouling accumulation (Coutts & Taylor, 2004; Murray et al., 2011). The presence of organisms, even biofilm, increases fuel consumption and decreases ships efficiency due to frictional drags and due to the frequency of application / removal of coatings and cleaning (Schultz et al., 2011; Dobretsov et al., 2013; Davidson et al., 2016).

A literature review was conducted on the Thomson Reuters Web of Science™ database in the same way done for ballast water and invasive species, now using the terms “biofouling” and “invasive species” (Figures 1.7 and 1.8). 184 manuscripts were found for the period from 2005 to 2017. All references apart from 34 were classified according to their main subject of research (category) as used for the previous search, whenever appropriate. In the present case, results showed a clear dominance of studies on the surveillance and monitoring of species in the researched period (33.3 %); followed by studies focussed on the development of techniques of treatments (21.3 %).



**Figure 1.7. Percentage of papers searched on Web of Science database using the expressions “biofouling” and “invasive species” according to the main subject of research for the period 2005 - 2017.**



**Figure 1.8. Number of papers per main subject of research per year for the period 2005 – 2017.**

From both reviews, either considering ballast water and invasive species or biofouling and invasive species, the two main subjects addressed by the published papers focussed on the surveillance and monitoring of species and on treatment techniques. Notwithstanding the fact that in the second review (biofouling and invasive species) the survey and monitoring of species studies were very predominant whilst for the expression ballast water and invasive species, treatment studies were slightly more representative than species surveillance and monitoring. In the latter case, results suggest that since the adoption of the IMO Convention in 2004 (before the searched period) and taking into account the performance standard required by the D-2 regulation of the Convention, more focus was given to the development of technologies of treatment to comply with it. Since there are no mandatory regulations in force for controlling biofouling but considering the known impacts of fouling species in key economic areas (e.g. water intake pipes, hydropower plants) more attention was given on the surveillance and monitoring of these undesired species. Possibly for accompanying and controlling its expansion, which naturally lead to the second topic more researched: treatment.

#### **1.4.1. Regulations on biofouling**

As set in the Agenda 21, Chapter 17, adopted during the 1992 Rio Conference on Environment and Development, States were called to take appropriate measures in order to reduce pollution caused by organotin compounds used in anti-fouling systems. In 1990 the International Maritime Organization Marine Environment Protection Committee had adopted a resolution recommending Governments to adopt measures to eliminate the use of anti-fouling containing tributyltin (TBT). In November 1999, an International Maritime Organization Assembly resolution called for a global prohibition on the application of organotin compounds used as biocides in anti-fouling systems on ships by 1 January 2003, and for a complete prohibition by 1 January 2008. In 2001, this instrument was

adopted as the International Convention on the Control of Harmful Anti-fouling Systems on Ships, which entered into force in September, 2008. Although this Convention did not focus on the spread of non-native species, it was indirectly related.

As a result of the work conducted by a working group on biofouling and invasive species at the International Maritime Organization, the problem was initially tackled through the adopting of a set of voluntary regulations. In 2011, the International Maritime Organization Marine Environment Protection Committee issued Resolution MEPC.207(62)), Guidelines for the control and management of ship biofouling to minimize the transfer of invasive aquatic species, outlining measures to minimise the risk associated with ship biofouling. Two subsequent sets of guidance on biofouling were adopted in the following years, one specific for recreational craft (MEPC.1/Circ.792, 2012) and another one with a view to assessing the implementation of the 2011 by country (MEPC.1/Circ.811, 2013) (Castro, 2014).

Following in the footsteps of the ballast water vector, it seems that the control of non-native species through biofouling will be the next International Maritime Organization Convention to be adopted in a near future.

## **1.5. Summary and thesis aims**

The spread of aquatic non-native species has been a subject of concern for scientists and regulators since the 1980s when human-mediated transportation, mainly related to shipping, was recognised as a major pathway for species transfer and spread. Increasing world trade and the resulting rise in shipping have brought more awareness on the issue, demanding a response from the international community to the threat represented by non-native marine species.



This thesis addresses the role of shipping as an unintentional and very efficient pathway for spreading aquatic non-native species, mainly phytoplankton, around the globe. More specifically, experiments conducted in this research focussed on the investigation of portable instruments developed to support inspections regimes to report on the efficiency of ballast water management systems required by international regulations in force (e.g. the International Maritime Organization Convention for the Control and Management of Ships Ballast Water and Sediments and the Standards for Living Organisms in Ships Ballast Water Discharged in U.S. Waters, 2012). Phytoplankton groups, in the size range defined by these international regulations, collected from natural assemblies as well as from treated ballast water samples collected on board ships equipped with ballast water management systems were analysed with regards their viability. With regards to the biofouling vector, experiments were conducted to report on the efficiency of using low salinity to kill fouling organisms in areas of the ship where it is difficult to coat and therefore tend to accumulate fouling organisms, e.g. ships sea-chest.

This thesis comprises seven chapters; a general introductory chapter on shipping and its importance for the economy and wealth of countries, as well as on its importance in the transfer of aquatic non-native species through ballast water and biofouling (Chapter 1). Chapter 2 focussed on invasive species in the North-eastern and South-western Atlantic Ocean, where shipping routes were investigated with regards to a possible interchange of species between the two regions. Chapter 3 presents a case of study on the application of a unilateral policy adopted in 2005 in Brazil for ballast water. Results of ten years of ballast water enforcement are presented and discussed as an example for the early years of implementation of a regulation. Chapter 4 is on the investigation of phytoplankton viability from samples collected during one year at L4 sampling site in the English Channel as well as from eight ballast water tests, using different fluorescence techniques. Chapter 5 examines the application of flow cytometry and fluorometry in characterizing natural

phytoplankton communities from the Wadden Sea, in the Netherlands and from L4, in the English Channel in the size range between 10  $\mu\text{m}$  and 50  $\mu\text{m}$ . In addition, the annual variability of cellular properties like cell size and chlorophyll fluorescence combined with the size distribution of the cells was investigated. Chapter 6 reports on the results of using low salinity for minimizing marine biofouling from ship sea-chest and on the importance of adopting simple measures to improve biosecurity. Finally, the last chapter discusses possible implications of the findings reported on the present thesis for the shipping industry and more specifically to the Ballast Water Management Convention and the Guidelines for the Control and Managements of Ships biofouling to minimize the transfer of Invasive Aquatic Species.

## **Chapter 2 : Invasive species in the Northeastern and Southwestern Atlantic Ocean: a review**

A version of this chapter has been published and is available online as:

Castro, M. C. T., Fileman, T. W., & Hall-Spencer, J. M. (2017). Invasive species in the Northeastern and Southwestern Atlantic Ocean: A review. *Marine Pollution Bulletin*, 116(1-2), 41-47.

DOI: 10.1016/j.marpolbul.2016.12.048

## **Abstract**

The spread of non-native species has been a subject of increasing concern since the 1980s when human-mediated transportation, mainly related to ships' ballast water, was recognized as a major vector for species transportation and spread, although records of non-native species go back as far as 16<sup>th</sup> Century. Ever increasing world trade and the resulting rise in shipping have highlighted the issue, demanding a response from the international community to the threat of non-native marine species. In the present study, we searched for available literature and databases on shipping and invasive species in the North-eastern (NE) and South-western (SW) Atlantic Ocean and assess the risk represented by the shipping trade between these two regions. There are reports of 44 species associated with high impacts for the NE Atlantic and 16 for the SW Atlantic, although this may be an underestimate. Vectors most cited are ballast water and biofouling for both regions while aquaculture has also been a very significant pathway of introduction and spread of invasive species in the NE Atlantic. Although the two regions have significant shipping traffic, no exchange of invasive species could be directly associated to the shipping between the two regions. However, it seems prudent to bring the exchange of ballast water between the two regions under control as soon as possible.

Keywords: Invasive species, NE Atlantic Ocean, SW Atlantic Ocean, ballast water, biofouling, risk assessment.

## **2.1. Introduction**

The spread of non-native species is a major threat to the biodiversity of the planet (Gurevitch & Padilla, 2014; Butchart et al., 2010; Firn et al., 2015). Humans cause the spread of marine species in various ways (e.g. vessel biofouling and the translocation of shellfish), with ballast water known to be one of the most important vectors for invasions

by non-native species (Carlton et al., 1995; Drake, 2015). Ballast water is used to adjust the draught and trim of a ship to improve manoeuvrability and stability with an estimated 3-10 billion tons of ballast water transferred globally each year (Gollasch et al., 2002; Tamelander et al., 2010). As about 80% of international trade, in terms of volume, is carried by sea, shipping routes connect coastal regions worldwide (UNCTAD, 2014). Ballast water was first suggested as a vector for non-native species dispersal more than 90 years ago (Hallegraeff & Bolch, 1992) and it is now considered to be one of the major threats to marine biodiversity (Ruiz et al., 2000; Takahashi et al., 2008; Masson *et al.*, 2013; Fowler & McLay, 2013).

The challenge with ballast water is that a myriad of organisms are transported across natural barriers before dispersal, transcending biogeographic regions. Ship's ballast tanks can carry about 3,000 – 7,000 species (Carlton & Geller, 1993; Gollasch et al., 2002; Carlton, 2001; Endrensen et al., 2004) and this wholesale movement of marine life contributes to the spread of disease; it disrupts coastal ecosystems and is causing the homogenization of coastal habitats (Ruiz *et al.*, 2000; Drake & Lodge, 2004; Rahel, 2007, Katsanevakis et al., 2014). The transfer of invasive species in ballast water is an international problem that is currently out of control; increasing shipping trade along with increasing ship size and speed, the opening up of new trade routes such as across the Arctic, man-made coastal habitat modification, the development of offshore windfarms and the global effects of sea-surface warming and acidification are all contributing to marine biological invasions (Williams et al., 2013; Allen & Hall-Spencer, 2015).

Alongside ballast water and biofouling on ships, non-indigenous species can also be introduced and spread by man-made structures at sea, canals, aquaculture activities and releases from aquaria. In many cases the spread of non-indigenous marine life is as a result of multiple pathways of introduction e.g. ballast water releases can be compounded

by those from biofouling on hulls, propellers, sea-chests and other niche areas (Ruiz *et al.*, 1997). These combinations of vectors can transport aquatic organisms at multiple life stages and include free-living as well as attached forms.

During the United Nations Conference on Environment and Development in 1992, known as the “Rio Earth Summit”, the spread of non-native species was recognized as one of the four greatest threats to biodiversity resulting in severe environmental, economic and public health impacts. This led to Article 8 (h) of the Convention of Biological Diversity which calls for the control and management of invasive species. The International Maritime Organisation (IMO) is a United Nations body that deals with the safety, security and environmental performance of international shipping. It has been working with member states to help control the spread of non-native species in ballast water and vessel biofouling. In 2000, the International Maritime Organisation launched their ‘Removal of Barriers to the Effective Implementation of Ballast Water Control and Management Measures in Developing Countries’ initiative, widely referred to as the ‘Global Ballast Water Management Programme’, and this led to the ‘Convention on the Control and Management of Ships’ Ballast Water and Sediments’. In September, 2016, after more than a decade of delay, this Convention has finally reached the requirements for entry into force.

According to a comprehensive review on the impact of non-native species on ecosystem services within Europe, the highest number of non-native marine species with described ecological and economic impacts was found in the eastern Mediterranean Sea and in the North Sea (Zenetos *et al.*, 2012; Vilà *et al.* 2009). In 2014, a pan-European review focused on non-indigenous marine species classed 87 species as ‘High Impact’ with seventeen of these species associated with only negative impacts, the majority (63) were documented as having both positive and negative impacts (Katsanevakis *et al.*, 2014). Among the

species with negative impacts on biodiversity is the gastropod *Rapana venosa* that feeds on bivalves and can decimate commercial bivalve stocks. Because of the high densities achieved, the crab *Hemigrapsus sanguineus* was described among the species with a negative impact on native species recruitment, e.g. some species of barnacles and mytilid bivalves (Katsanevakis et al. 2014). On the other hand, some species are able to interfere positively in the biological process and may act as a control over other invasive species, e.g. *Crepidula fornicata* that is able to “cause a shift of phytoplankton blooms from toxic flagellates to diatoms” (Thieltges et al. 2006), besides some species known as ecosystem engineering usually associated with both positive and negative impacts, like most macroalgae (Katsanevakis et al., 2014). The problems associated with the spread of non-native marine organisms in Europe are tackled in regional agreements. The Barcelona Convention for the Protection of the Mediterranean Sea against Pollution, originally adopted in 1975 and replaced in 1995, includes invasive species monitoring as one of the key priorities for the next decade and in 2005 adopted an action plan concerning species introductions and invasive species in the Mediterranean Sea; the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention), and the Convention on the Protection of the Marine Environment of the Baltic Sea Area, known as HELCOM area (Helsinki Convention) both from 1992, adopted in 2008 joint guidelines with a view to minimizing the risks of introduction and spread of non-native species in the North-East Atlantic and in the Baltic Sea. These agreements have led to action plans and initiatives designed to tackle the ever increasing introduction of non-native species in European seas and in the NE Atlantic.

Unlike the NE Atlantic, South American countries do not have ongoing projects or comprehensive studies on non-native marine species. In a review of non-native marine species along the coast and shelf areas off Argentina and Uruguay, Orensanz et al. (2002) listed 31 introduced species, whereas 58 non-native marine species are known to have

been introduced along the coastline of Brazil, nine of which categorized as invasive (MMA, 2009). Species classed as invasive for the SW Atlantic were mostly zoobenthos, e.g. *Tubastraea coccinea*, *Isognomon bicolor* and *Styela plicata*. A big problem in the region was caused with the introduction of the golden mussel (*Limnoperna fortunei*), presumably from ballast water tanks. Native from rivers in China and in South-East Asia, this freshwater species invaded South America through the La Plata basin during the 1990's (first record was in 1991, Pastorino et al., 1993) and caused great economic and ecological problems once it is able to attach themselves to any sort of substrates including the settlement on native mussels. Economic losses have been significant since their spreading within South America, where they are known to cause damage in water distribution systems (Darrigran et al., 1999).

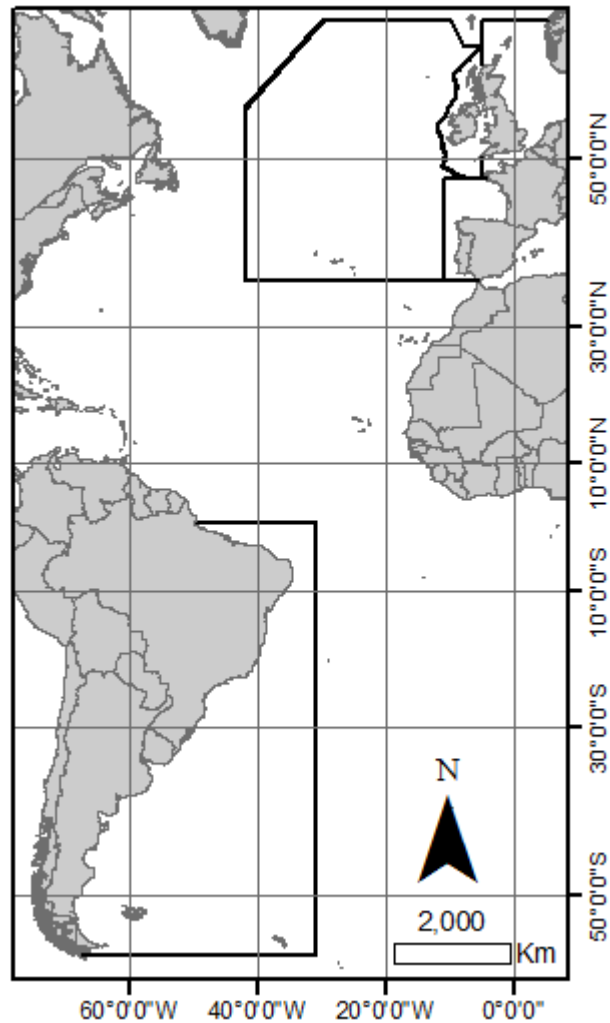
The aim of this study is to assess the amount of shipping that takes place and to update published data on non-native marine species that have become invasive in the North East and the South West Atlantic Ocean. These two areas were chosen since the two regions may be exchanging non-native species taking into account the significant shipping trade between the two regions.

## **2.2. Methods**

### **2.2.1. Area of study**

This study is focussed on the NE and SW Atlantic Ocean. Our NE Atlantic region borders the four non-Arctic areas delimited by the OSPAR Commission, namely; the Greater North Sea (II), the Celtic Seas (III), the Bay of Biscay and Iberian Coast (IV) and the Open Ocean (V). Our SW Atlantic region extends south of the Equator to 55° 00' S and out from the South American continent to 20° W (Figure 2.1).





**Figure 2.1. Study area: the NE Atlantic Ocean and the SW Atlantic Ocean.**

### **2.2.2. Shipping trade data**

We assessed the amount of shipping traffic using the United States Coast Guard's Automated Mutual-Assistance Vessel Rescue System website ([www.amver.com](http://www.amver.com)). We constructed a shipping density plot to calculate the amount of traffic in June 2013, as this was the most recent month with comprehensive data available.

Data on shipping of all cargo types between Brazil and the fifteen OSPAR countries were obtained from the Brazilian National Waterways Transportation Agency website

([www.antaq.gov.br](http://www.antaq.gov.br)) through the 'estadística' link for the year 2014. Data on shipping trade between Argentina and the OSPAR countries were taken from the Argentinian National Institute of Statistics website (<https://opex.indec.gov.ar/>). Finally, for Uruguay, these data were obtained from the websites [www.uruguayxxi.gub.uy/](http://www.uruguayxxi.gub.uy/) and <http://www.tradingeconomics.com/> which reports on trade for the year 2014.

### **2.2.3. Invasive species data**

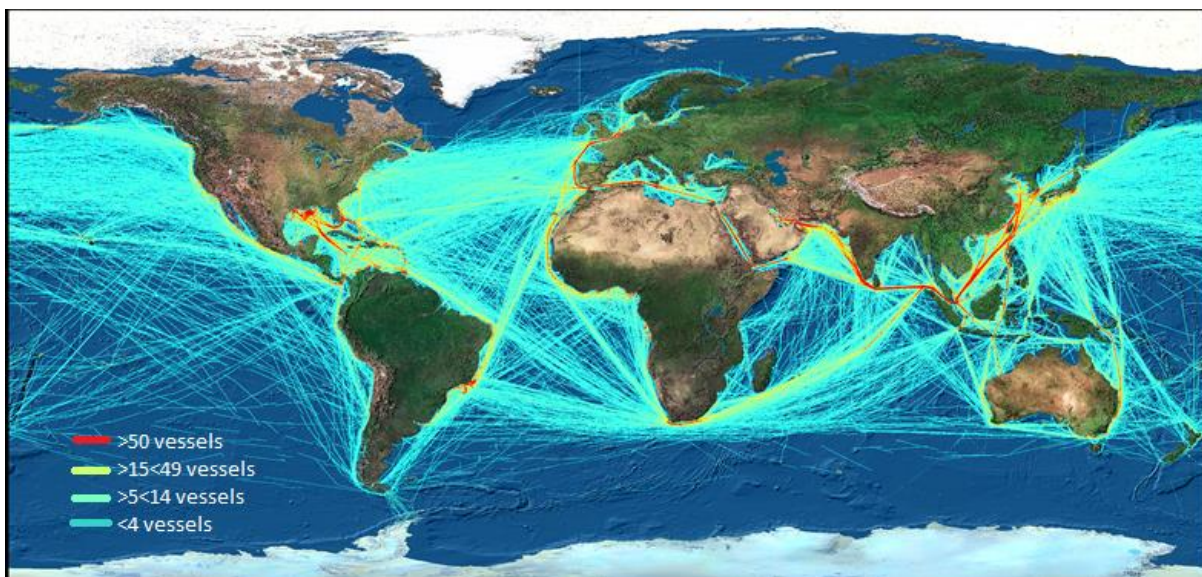
In November 2015, we collated a list of marine and brackish invasive species for our study areas in the NE and SW Atlantic Ocean. We only included non-native species that are known to have had high impacts. For instance, when searching the European Alien Species Information Network the categories we selected were “marine”, “oligohaline”, “high impact” and “alien” for our study areas. The species highlighted by this process were then searched for in the World Register of Marine Species (WoRMS) (<http://www.marinespecies.org/index.php>) to augment the information available. During this iterative process the following databases were consulted: the Global Invasive Species Database (GISD) (<http://www.issg.org/database/welcome/>), the European Network on Invasive Alien Species (<http://easin.jrc.ec.europa.eu/>), Delivering Alien Invasive Species Inventories for Europe (<http://www.europe-aliens.org/default.do>), the European Register of Marine Species, the Information System on Aquatic Non-indigenous and Cryptogenic Species (<http://www.corpi.ku.it/databases/index.php/aquanis>), the GB Non-native Species Secretariat (<http://www.nonnativespecies.org/home/index.cfm>), the North Atlantic Register for Marine Species (<http://www.vliz.be/vmdcdata/narms/>), AlgaeBase (<http://www.algaebase.org/>) and FishBase (<http://fishbase.org/search.php>). A recent review by Katsanevakis et al. (2014) for the European seas was also used to cross check and augment our database.

For the SW Atlantic Ocean, our main data sources were reviews organized by Lopes (MMA, 2009) for Brazilian coastal waters and by Orensanz et al. (2002) for coastal and shelf areas off Argentina and Uruguay. In addition, we consulted the following studies: Genzano et al. 2006; Darling et al., 2008; Ignacio et al; 2010; Irigoyen et al., 2011; Lages et al., 2011; Ferrapeira et al., 2011; Guadalupe Vázquez et al., 2012; Sant'Anna et al., 2012; Sylvester et al., 2013; Bonel et al.; 2013; Rocha et al., 2013; Riul et al., 2013; Rechimont et al., 2013; Guinder et al., 2013; Schwindt et al., 2014; Freire et al., 2014; Moreira et al., 2014; Marques & Breves, 2014; Altvater & Coutinho, 2015; Ferreira et al., 2015; Carlos-Junior et al., 2015; Sant'Anna et al., 2015. We also searched WoRMS, GISD, National Exotic Marine and Estuarine Species Information System (<http://invasions.si.edu/nemesis/index.jsp>), the Exotics Guide (<http://www.exoticsguide.org/>), the Invasive Species Compendium (<http://www.cabi.org/isc/>) and the Conservation Gateway from the Nature Conservancy Global Marine Invasive Species database (<https://www.conservationgateway.org/ConservationPractices/Marine/Pages/marineinvasives.aspx>).

## **2.3. Results**

### **2.3.1. Shipping trade data**

A snapshot of shipping traffic for June 2013 shows the major world shipping routes (Figure 2.2) and the numbers of ships travelling in and out of NE and SW Atlantic ports.



**Figure 2.2. World marine traffic density plot for June 2013 (data from the US Coastguard Automated Mutual-Assistance Vessel Rescue System website, [www.amver.com](http://www.amver.com)).**

In 2014, ca 100 million tonnes of cargo was exported from Brazil to Europe, down 7% on the previous year. Around 31 million tonnes of cargo were exported from Europe to Brazil in 2014, up 6.4% on 2013 (ANTAQ, 2015). The amount of cargo exported from Brazil to OSPAR countries was ca 80 million tonnes in 2014 (mostly bulk solids), with main destination ports in the Netherlands, France and Spain. In the opposite direction the total reached about 18.5 million tonnes (mostly container vessels), embarked mainly in ports of Spain, Belgium, the Netherlands and German.

In 2014, around 8.5 million tonnes of cargo were exported from Argentina to OSPAR countries, mainly to the Netherlands, Spain and the United Kingdom whereas Germany was the source of most of her OSPAR imports (INDEC - Dirección Nacional de Estadísticas del Sector Externo - <https://opex.indec.gov.ar/>). That year almost 800,000 tonnes were exported from Uruguay to the OSPAR countries, mainly to Portugal and Spain with the latter being the main source of OSPAR country shipping imports to Uruguay ([www.uruguayxxi.gub.uy/](http://www.uruguayxxi.gub.uy/)).

Nevertheless, taking into account the total cargo exported by the three South American Countries in 2014, the amount of cargo exported to OSPAR countries represented a small fraction from the total (Table 2-1).

**Table 2-1. South American exports to OSPAR Countries and total amounts exported in 2014.**

Countries	The Netherlands	Spain	France	UK	Portugal	Total exported in 2014 (T)
Argentina	3%	2%		2%		83,134,257
Brazil	3.5%	1%	1%			968,872,333
Uruguay		1%			4%	13,866,907

### 2.3.2. Invasive species data

A search of the EASIN network for records associated with high impact marine and oligohaline non-native species returned >100 species for our NE Atlantic study areas. From that, 49 species are documented as high impact invasive species to the NE Atlantic region (Table 2-2). For instance, we excluded from this review the amphipod *Dikerogammarus villosus*, native to Ponto-Caspian region as it is mainly a freshwater species with very low salinity tolerance range. We also excluded *Mytilus edulis*, the common blue mussel, since it is considered native to the NE Atlantic (Wonham, 2004) and its invasiveness status is under discussion for the SW Atlantic Ocean (Savoya et al., 2013).

**Table 2-2: Invasive marine and oligohaline species recorded for the NE Atlantic Ocean (Annex 1)**

For the SW Atlantic Ocean, it was possible to list 16 species to which well documented high impact were described. The list of invasive marine and oligohaline species recorded for the SW waters is presented in Table 2-3.

**Table 2-3: Invasive marine and oligohaline species recorded for SW Atlantic Ocean (Annex 2)**

Both tables present phytoplankton species classified according to their class or infraphylum (dinoflagellates) while species of plants and animals are classified according to the phylum, as verified in the World Register of Marine Species (WoRMS).

## **2.4. Discussion**

We have shown that there is a significant volume of shipping traffic between the NE and the SW Atlantic Ocean; cargo is mainly moved in the north-eastern direction and ballast water is mainly moved south-west, which presents a higher risk to export ports and terminals where higher volumes of discharge occur. Propagule pressure is a crucial factor that affects the establishment of non-native species and it is dependent on the number of organisms released and on the number of release events (Lockwood et al., 2005). Propagule pressure can vary according to ship type, size and speed. Large ships with higher volumes of water in their ballast tanks have increase survival of transported organisms and more species are discharged. A good example is the mineral ore shipping trade, which accounted for the majority of goods exported from Brazil to the OSPAR area in 2014; almost 80% of the total (ANTAQ, 2015). Pereira (2012) estimated volumes between 10,000 to 120,000 m<sup>3</sup> of ballast water discharged into Brazilian coastal areas per journey due to the exportation of iron ore.

The shipping pressure is also revealed by the direction each ship takes. We found that the cargo exportation in the NE direction was about 90 million tonnes in 2014, around four times higher than in the opposite direction. From the perspective of possible non-native species introductions and propagule pressure, it represents a higher risk to the SW Atlantic assuming that ships are loading cargo (and, therefore, unloading ballast water) and unloading it in the NE coast of Europe (and, therefore, taking on ballast water). However, the number of invasive species' records for the region doesn't reflect this

assumption which is probably related to differences in the salinity of the donor and recipient ports.

Other important aspect to be considered in a risk assessment is related to ships' route. In our case the main SW-NE route has the Netherlands as the main source of ballast water. It may mean that species recorded for ports in the Netherlands (e.g. Rotterdam) are more likely to be introduced in ports located in the SW Atlantic Ocean. However, previous surveys in the SW Atlantic input a higher risk to routes whose final destinations are located in the Indo-Pacific region, since these account for around 30% of non-native species found in the SW Atlantic (MMA, 2009) possibly reflecting a secondary introduction from a primary site of introduction. Besides ships' influence over the ballast water discharges, there are other aspects that might interfere following discharge and act directly on the species' ability to colonize a new environment. Aspects like the similarity between the places where the water was taken and where it was unloaded, interspecies competition and species-specific tolerances to abiotic factors are crucial in the settlement of non-native species in new environments (Lockwood et al., 2005; Lockwood & Somero, 2011).

Results from the databases and scientific literature researched showed 44 non-native species associated with high impacts for our NE Atlantic while 16 were identified in the SW Atlantic. Species described at least in one database or by an author as freshwater and oligohaline or brackish were included in the present review. Therefore, the two bivalves *Dreissena polymorpha* and *Limnoperna fortunei*, as well as the salmonid *Oncorhynchus mykiss* were included.

In terms of introduced species recorded to European seas and the NE Atlantic, many studies and reviews can be found in the scientific literature. In that respect and according to a recent review, an updated inventory of 87 non-native marine species in European Seas was proposed in 2014, including those species with a documented high impact on

ecosystem services or biodiversity (Katsanevakis et al., 2014). When comparing the present list (Table 2-2) to the one proposed by Katsanevakis et al. for the Celtic and the North Seas, common areas in both studies, most species are present in both lists. The only exceptions are the amphipod *Chelicorophium curvispinum*, a freshwater / oligohaline species with a salinity tolerance of up to 6 (Van den Brink et al., 1993), and the euryhaline crabs *Rhithropanopeus harrisii* and *Hemigrapsus takanoi*. A dominance of the brush-clawed shore crab over the native European green crab (*Carcinus maenas*) was found in a study along the French coast and in the North Sea (Dauvin et al., 2009). It is worth noting that up to 2005, the name *Hemigrapsus penicillatus* was used for two crabs that are now known to represent two distinct species (*Hemigrapsus penicillatus* and *H. takanoi*). From the invasive species compendium, it is observed that *H. takanoi* was only recently described; therefore first records for Europe, which date back from the mid-1990s, were named *H. penicillatus* (Asakura et al., 2008; Yamasaki et al., 2011). Another similar species *H. sanguineus* is recorded as invasive to Europe and included in Katsanevakis et al. review and in the present study. From the phytoplankton, three species are recorded in Table 2-2.

In the SW Atlantic Ocean, including Brazilian waters, a comprehensive review compiled by Lopes (MMA, 2009) found 58 marine species recorded as non-native, nine of which have invasive status, within them, two phytoplankton species. A couple of years later, another study along the Brazilian coast increased the known number of non-native species in the region. A total of 343 benthic invertebrate species were recorded (65% non-native and 35% cryptogenic) (Ferrapeira et al., 2011). Nevertheless, an apparent overestimation in the species numbers was highlighted by Rocha et al. (2013) due to some mistakes mainly related to species taxonomy and geographic distributions. For Uruguay and Argentinian coasts, the review conducted by Orensanz et al. (2002) described 31 species with a well-documented exotic status, plus 46 species with a cryptogenic status.



The blue crab (*Callinectes sapidus*) is recorded as a non-native species with high impact in the NE Atlantic Ocean has its natural range described along the western Atlantic coast from Nova Scotia to Argentina (Milliken & Williams 1984; NWARMS). Ballast water is the main vector associated to its introduction outside its native range (Katsanevakis et al, 2012). *Ciona intestinalis* has been identified on both sides of the North Atlantic and has been noted to have spread to the west coast of North America, South America, Australia, New Zealand, Asia and Africa (Kott, 1990; NIMPIS, 2002; Lambert & Lambert, 2003 apud Therriault & Herborg, 2008b). Nevertheless, the native range of *C. intestinalis*, for instance, is a focus of continuing debate (Therriault & Herborg, 2008a).

From the total of 16 species in the SW Atlantic, the diatom *Coscinodiscus wailesii* is recorded in Brazilian waters as an invasive species known to be associated with dense blooms causing losses to fisheries and aquaculture (MMA, 2009). This species is described as euryhaline and eurythermal and tolerant to high concentrations of heavy metals (Proença & Fernandes, 2004; Rick & Dürselen, 1995) and has its native range normally linked to the North Pacific. Described vectors of introduction are ballast water and aquaculture activities. To the NE Atlantic, the diatom has a recorded range from France to Norway. However, Gómez (2008) described periods of high abundances of *C. wailesii* connected to unusual climatic conditions, being the species currently restricted to residual populations during the winter (Boalch, 1987; Edwards et al., 2001 apud Gómez, 2008).

The dinoflagellate *Alexandrium tamarense*, usually considered a harmful species (associated to Paralytic Shellfish Poisoning toxins) is native in the North Atlantic Ocean as well as in the North Sea. On the other hand this species is considered invasive to the SW Atlantic Ocean where it is widely spread (Table 2-3).

Species listed as invasive in both regions of study are *Spartina townsendii* var. *anglica*, (native of southern England) *Ficopomatus enigmaticus* (native range described as

unknown), *Magallana gigas* (NW Pacific Ocean) and *Bugula neritina* which is a cosmopolitan species. From those, the common cord-grass (*Spartina anglica*) is described as a hybrid from one species native to North America (*Spartina alterniflora*) and other from Europe (*Spartina maritima*). The resultant species is now considered native to southern England and first introductions of *Spartina alterniflora* in Europe in the late 1870s are associated with ballast water while later introductions of the hybrid were intentional for coastal protection purposes (Nehring & Adersen, 2006).

The recorded invasive species' list for the NE does not present any native species that exclusively inhabit the SW Atlantic Ocean. Species like the Australian tubeworm, *Ficopomatus enigmaticus*, has its native range associated with the Southern Hemisphere and, in some records to the eastern coast of South America. Nonetheless, it is also recorded as an introduced species to Argentina (WoRMS and DAISIE databases). The amphipod, *Platorchestia platensis*, originally described to Uruguay had its native range updated to unknown (Jensen, 2010). Finally, the ctenophore *Mnemiopsis leidyi* has its native range described for the Atlantic Coast of North and South Americas (Costello, 2001).

Regarding the vectors involved in the transport of non-native species, for the NE Atlantic ballast water is the most cited vector of introduction / dispersion whether alone or as one of the possible vectors / pathways, present in almost 48% of the records. In some records, however, the pathway of introduction was identified as "shipping", probably referring to both ballast water and biofouling vectors, which might increase ballast water as the main vector of introduction to more than 65% of the total. Applying the same reasoning for the biofouling vector, the latter reaches almost 55% of the records as preferential vector of introduction / spread. In sequence, biofouling and aquaculture activities (around 38% each) are the most cited while natural dispersion was cited as the pathway in six of

records. For the SW Atlantic, ballast water was cited in thirteen records as the most probable vector of introduction alone or together with other vectors or pathways (73%) followed by biofouling (47%).

Successful establishment of exotic species in a new environment is usually highly associated with the environmental similarity between the donor and the receptor areas (Keller et al., 2011). This reduced the chance of exchange for the tropical regions of the SW Atlantic although the temperate regions are more at risk from invasion by non-native species from Europe.

## **2.5. Conclusion**

This review updates information on invasive species recorded for the NE and SW Atlantic Ocean. The number of non-native species that have become invasive with high ecological impacts are 44 in the NE Atlantic and 16 in the less well studied south-western Atlantic. The main vector of introduction and spread of these invasive species is shipping (both ballast water and biofouling). Aquaculture is also an important pathway of introduction, particularly in the NE Atlantic. However in most cases where more than one vector and pathway were cited it is difficult to disentangle the level of influence of the different vectors and/or pathways. In many cases a combination of vectors may carry species at multiple life stages. No clear evidence for the exchange of species between the NE and the SW Atlantic has been noted in this review, although secondary introductions from primary sites of introduction are highly possible and, therefore, this does not negate international efforts that are underway to improve biosecurity. Phytoplankton species recorded in both areas are associated with fisheries and aquaculture losses. Due to the fact that they are small, capable of forming cysts and deal with prolonged periods of darkness, they have better chances to survive in ballast tanks, deserving more effort in terms of research (McCarthy and Crowder, 2000).

The development of scientific research and case studies focused on non-native marine species' vectors as a whole instead of focusing on individual species or individual vectors seems to be a way forward with a view to avoiding impacts and associated losses and costs (Williams et al., 2013). Integrated studies might be the best way to produce valuable forecasts of ecological and economic importance of invasion on ecosystems around the world (Ibanez et al., 2014). Non-native species are certainly a crucial issue that needs to be addressed to raise general awareness and publicity, alongside scientific surveys and monitoring, improved data availability, regulations (preferably international ones in order to avoid legal uncertainties), management tools, risk assessment, stakeholders' commitment, enforcement, best practices and constant surveillance (Costello et al., 2007; Williams et al., 2013; Lehtiniemi et al., 2015).

### **Chapter 3 : Ten years of Brazilian ballast water management**

A version of this chapter has been published and is available online as:

**Castro, M. C. T.**, Hall-Spencer, J. M., Poggian, C. F., & Fileman, T. W. (2017). Ten years of Brazilian ballast water management. *Journal of Sea Research*, 133, 36-42.

DOI: <http://dx.doi.org/10.1016/j.seares.2017.02.003>.

## **Abstract**

In 2005, Brazil addressed the environmental challenges posed by ballast water through a unilateral regulation, called the Maritime Standard Nº 20 (NORMAM-20), applied to all shipping in her waters. This world-leading decision was the culmination of a process that started during the 1990's. Here, we summarize how these ballast water regulations were brought in and adopted and present the findings of 10 years of enforcement (2005-2015) in 39 ports along the Brazilian coast. We show that compliance with the Brazilian standard has increased significantly since the regulations were implemented ( $p < 0.001$ ). After five years of implementation, non-compliance decreased probably reflecting an increase in awareness of the Brazilian Standard and a shift in the shipping industry commitment to minimise and control the spread of invasive species through ballast water. The Brazilian experience shows that very high levels (97%) of compliance with ballast water management regulations can be made to work in a region of global importance to the maritime industry. In the last decade, the rules governing ballast water in Brazil have evolved to address the demands from the maritime community and to provide updates such as imminent requirements for the use of ballast water management systems on board ships. These regulations are rarely cited when ballast water regulations are discussed internationally, yet there is much to learn from the proactive approach taken by Brazil such as what is feasible and enforceable.

**Keywords:** Shipping, biosecurity, invasive species, marine biology, international trade.

### **3.1. Introduction**

Today, about 80% by volume of international trade is carried by sea along shipping routes that connect coastal regions worldwide (UNCTAD, 2014). The shipping industry has played a very important role in the development of economies around the world; increasing industrialization and changes in the world economies have fuelled the trade and set a growing demand for consumer products and advances in shipping technology mean that has become an increasingly efficient and a swift method of transport (IMO, 2012). However, shipping activities need environmental controls to help avoid accidents, to curb pollution and inhibit the transfer of organisms across biogeographic boundaries (Leal-Neto, 2007).

Ballast water is taken on board ships to improve manoeuvrability, stability and safety and is of major environmental importance since when it is discharged it can spread non-native harmful aquatic organisms and pathogens able to impair the environment, human health, biological diversity among others (IMO, 2004). Descriptions of non-native species associated with shipping date back to the 16<sup>th</sup> Century with a scientific focus on the problem building up through the 1970s leading to Canada and Australia raising the risks posed at meetings of the International Maritime Organization (Galil et al., 2009).

Shipping is the main source of unintentional transfer of organisms, including pathogens, via ballast water discharges and biofouling (Ruiz et al., 2000; Bax et al., 2003; Coutts & Taylor, 2004; Drake & Lodge, 2007; Takahashi et al., 2008). International initiatives have been taken to avoid the transference of non-native species through ballast water, initially with the adoption of voluntary guidelines which recommended the ballast water exchange in mid-ocean as a management option (International Maritime Organization (IMO) Marine Environment Protection Committee Resolution MEPC.50(31), IMO Assembly Resolution A.774(18), IMO Assembly

Resolution 686(20)). In 2004, the International Convention for the Control and Management of Ships Ballast Water and Sediments (BWM Convention) (IMO, 2004) was adopted by consensus. Nevertheless, its adoption has raised many important discussions, including its enforcement (beyond the documental stage of inspection) and this might led to the prolonged delay in ratifying the Convention, that only recently reached the 35 per cent of world merchant shipping tonnage, as provided in article 18.

From a national perspective, there have been many major environmental and economic problems associated with the introduction and spread of the golden mussel (*Limnoperna fortunei*) during the 1990's (i.e. agglomeration and clogging inside cooling systems and discharge pipes, deterioration and obstruction of filters at Itaipu binacional hydroelectric power plant) (Mansur et al., 1999; Danrigran & Drago, 2000). This led Brazil to unilaterally adopt her own ballast water management regulations to minimise the threat posed by invasive species.

### **3.1.1. Brazilian maritime administration and ballast water management**

The Brazilian Maritime Authority enforces, under naval command, national and international regulations in waters under national jurisdiction and carries out inspections for the protection of human life, the safety of navigation and the prevention of environmental pollution. The Maritime Authority has a main Directorate of Ports and Coasts that oversees the implementation and enforcement of maritime regulations carried out by Port State Control Officers (PSCO).

National regulations on ballast water began in 2000 with the adoption of the Brazilian Maritime Authority's Standard nº 08 (NORMAM 08), superseded by reviews in 2013 and 2015 (Brazil, 2015). This required that each vessel in Brazilian territorial waters to send a completed Ballast Water Form to the local Port Captaincy and that a copy was shown during Port State Control inspections. In 2001, the Brazilian National Health Surveillance Authority imposed a similar requirement (Resolution RDC nº 217) (Brazil,



2001) to limit public health problems associated with ballast water with epidemiological surveillance and vector control at Sanitary Control Ports. This followed the occurrence of a small cholera outbreak in Paranaguá Bay, southern Brazil, in 1999, where the disease had never previously been reported (Riviera et al., 2013). Regulations for health surveillance made the Ballast Water Form mandatory for granting entry to ships into Brazilian ports. The resolution raises the possibility of sampling of ballast water tanks for identifying the presence of pests and pathogens and to verify physical and chemical parameters, at the Sanitary Authority's discretion (article 28). In December, 2009, this sanitary rule was updated by Resolution RDC nº 72 (Brazil, 2009).

In 2005, after a period of discussions with the Brazilian maritime community, the Director of Ports and Coasts adopted the Brazilian Maritime Standard for ballast water management (NORMAM-20) which stipulates obligations to ships and/or their agents including filling out and sending the Ballast Water Form and providing information about the ballast water handled by the ship and its management, mainly through the mid-ocean exchange (Castro et al., 2010). In 2014 the rule was revised and providing information about ballast water management systems has become compulsory (Brazil, 2014).

Taking into account the additional task on ballast water and considering the nature of the inspection (not merely documental), during the period between the adoption of the Brazilian Standard (June, 2005) and its entry into force (October, 2005), PSCO located along the coast were trained by specialists on ballast water, senior inspectors and ship's masters. Moreover, informative material and presentations on the new requirements were also delivered to ship owners and maritime agents, with a view to discuss and clarify any aspects associated with the adoption of the new ballast water requirements.

Inspection of ballast water is generally conducted during ordinary inspections by Port State Control officers and is based on documents required by the Brazilian ballast

water regulations. Data verified and collected by Port State Control officers are related to the ballast water management minimum requisites, the management practices adopted by the ship and on salinity tests conducted on board during the inspection.

Here we consider the Brazilian experience of ballast water management over the past decade, based on ballast water inspection reports (Fig. 3.1). During the period, Brazilian port State control officers verified vessel compliance to the national standard and reported back the results to the Brazilian Maritime Authority. Additional important initiatives taken concerning ballast water issues within the Country are also reported to describe how compliance is assessed.

## **3.2. Methodology**

### **3.2.1. Design and Area of study**

We considered 11,183 vessels in 39 ports / terminals (ANTAQ, 2016) aboard which naval inspections were carried out by the Brazilian Port State Control Officers during the period between 2005 and 2015. These ports / terminals are distributed along seven (of nine) Naval Districts according to the criteria adopted by the Brazilian Navy. Areas 1 to 7 cover the following ports and/or terminals:

Area 1: Ports / terminals of Rio de Janeiro, Angra dos Reis / Itacuruçá, Itaguaí / Sepetiba, Vitória, Praia Mole / Tubarão, Ponta de Ubu, Barra do Riacho / Portocel;

Area 2: Ports / terminals of Aracaju, Salvador;

Area 3: Ports / terminals of Fortaleza, Recife, Natal / Termisa, Suape, Pecém, Paracuru, Mucuripe, Maceió, Cabedelo, Areia Branca;

Area 4: Ports / terminals of Itaquí, Alumar, Belém, Ponta da Madeira, Fazendinha / Santana, Vila do Conde, Macapá;

Area 5: Ports / terminals of Rio Grande, Imbituba, Itajaí, São Francisco do Sul, Paranaguá, Antonina, Navegantes, Porto Alegre, Tramandaí, Santa Clara;

Area 6: Ports / terminals of São Sebastião, Santos;

Area 7: Port of Manaus.

### **3.2.2. Data collection**

Data used in the present study were collected from Port State Control reports on ballast water, which is divided into 17 fields, where general information about the ship and description of non-conformities are required. We analysed data related to ship compliance with the Brazilian standard, mainly reported in fields 14 and 15 (Figure 3.1). Compliance data were defined as the outcome variable and were categorised as a binary variable (compliant and non-compliant). This variable was distributed considering two periods of time (T): T1 from 2005 to 2010 and T2 from 2011 to 2015.

### **3.2.3. Data analysis**

Chi-Square tests were applied to assess differences in compliance between T1 and T2. Then, a binary-logistic regression (not adjusted) was conducted to test the effect of T1 and T2 on compliance with the Standard. A p-value < 0.05 was adopted as the statistical significance.

All analysis were fitted with IBM SPSS Statistics software (Version 22).

**REPORT OF THE IMPOSITION OF A CONTROL AND COMPLIANCE MEASURE TO ENHANCE MARITIME SECURITY  
BRAZILIAN MARITIME AUTHORITY NORM FOR THE MANAGEMENT OF SHIPS' BALLAST WATER (NORMAM-20/DPC).**



**(DIRECTORATE OF PORTS AND COASTS)**  
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Rio de Janeiro – RJ – Brasil  
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Telephone: (55) (21) 2104-5678  
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Copy to: Master  
Head Office  
PSCO

1. Name of reporting authority: <b>Directorate of Ports and Coasts</b>	2. Date of inspection: _____
3. Place of inspection: _____	
4. Name of the ship: _____	5. Flag of the ship: _____
6. Type of ship: _____	7. Call sign: _____
8. IMO number: _____	9. Gross tonnage: _____
10. Year of build: _____	
11. Ballast Water Management Plan - Recognize Organization : _____	
12. Last port of call: _____	
13. Last port of ballast: _____	
14. Non - Conformities	
<input type="checkbox"/> Ballast Water Management Plan Not Available	<input type="checkbox"/> Not complying with Brazilian Maritime Authority Norm Directions (NORMAM-20/DPC)
<input type="checkbox"/> Ballast Water Reporting Form Not Available	
<input type="checkbox"/> Fails in fulfilling Reporting Form	<input type="checkbox"/> Others
15. Description of Non - Conformity	
_____	
_____	
_____	
_____	
16. Specific control measures	
<input type="checkbox"/> None	<input type="checkbox"/> Fine
<input type="checkbox"/> Cargo operation modified or stopped	
<input type="checkbox"/> Ship ordered to leave the port for Ballast operation	<input type="checkbox"/> Notice
<input type="checkbox"/> Ship detained	
17. Issuing Office _____	Stamp _____
Port State Control Officer	
Name: _____	
Stamp: _____	
Telephone / fax: _____	_____ Master

Figure 3.1. Port State Control Report on Ballast Water.

### 3.3. Results

#### 3.3.1. Port State control reports

Overall compliance with Brazilian ballast water regulations is shown in Table 3-1. These data were collected aboard 11,183 ships inspected in Brazilian waters between 2005 - 2015.

**Table 3-1. Ships' compliance with the Brazilian Maritime Standard between 2005 and 2015 (per area of study).**

Area	Total of Ships	Compliant Ships (C)	Non-compliant Ships (NC)	% of Compliant Ships
1	2514	2454	60	97.6
2	206	191	15	92.7
3	1759	1703	56	96.8
4	3464	3407	57	98.4
5	2356	2259	97	95.9
6	841	811	30	96.4
7	43	41	2	95.3
Total	11183	10866	317	97.2

From the total of ships inspected during the period (Table 3.1), a clear predominance of inspections occurred in area 4 (31%) followed by areas 1 (22.5%) and 5 (21%); Port of Manaus, Amazonas had the lowest number of inspections (0.4%).

With a view to recognizing whether compliance with the Brazilian standard had varied in the first decade of implementation, we analysed the data in two time periods. Regional variations are shown in Figure 3.2 whilst a decrease in the proportion of non-compliant ships in T2 is shown in Figure 3.3.

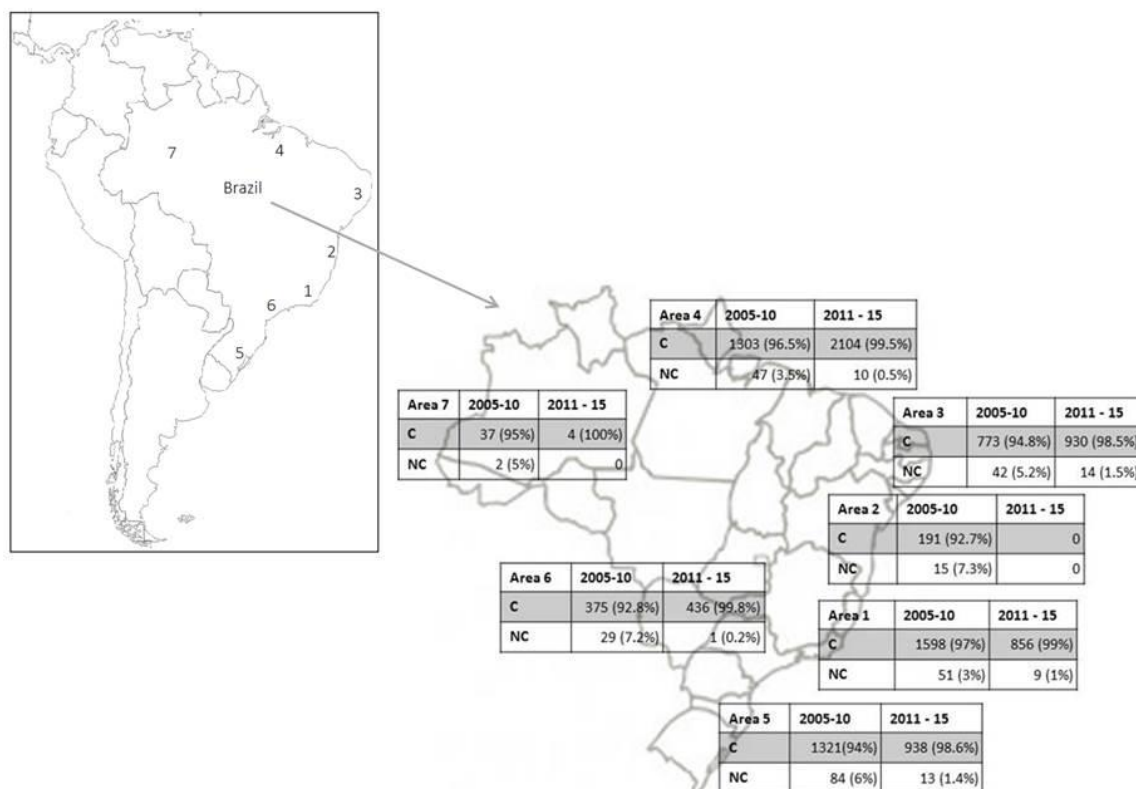


Figure 3.2. Regional variation in compliance with Brazilian ballast water regulations between 2005-10 and 2011-15.

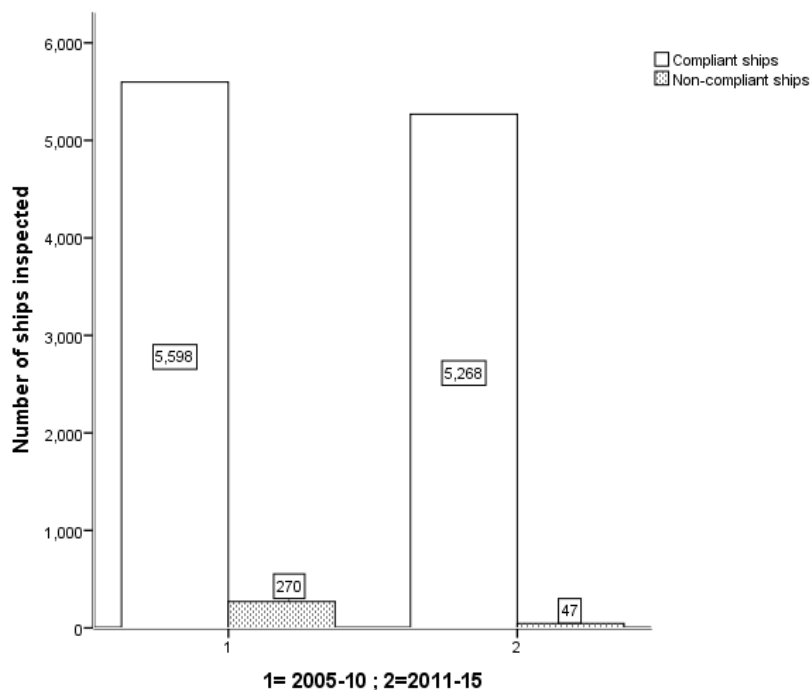


Figure 3.3. Increase in compliance with Brazilian ballast water regulations between 2005-2010 and 2011-2015.

Chi-Square tests ( $p < 0.001$ ) revealed a significant decrease in the overall proportion of non-compliant ships, and these differences were also significantly different in all areas ( $p < 0.001$ ) except 2 and 7 where few inspections were conducted. Results from a binary-logistic regression (not adjusted) showed a negative association between time and the number of non-compliant ships, meaning that with an increase in the number of years of inspection, a lower number of non-compliant ships was expected ( $\text{Exp}(\beta) = 0.185$ ;  $\text{SE} = 0.159$  and  $p < 0.001$ ).

### **3.4. Discussion**

The first results of Port State Control enforcement procedures in Brazilian Jurisdictional Waters were presented in 2009 (Castro & Poggian, 2009). After that, a more recent evaluation of Naval Inspection reports showed that from October 2005 to May 2012, the number of non-compliant vessels had decreased gradually, reaching values below 5% of the total number of inspected ships (Poggian, 2014). Here we investigated how compliance with the Brazilian standard has changed and show a significant rise in compliance between 2005-10 and 2011-15 ( $p < 0.001$ ) across all areas in Brazil, except for ports in Aracaju, Salvador and in Manaus where few inspections took place. Results obtained with the logistic regression confirmed this decrease, highlighting the positive effect of time over the number of compliant ships inspected in Brazilian ports / terminals.

Discrepancies in the number of inspections within the Country (as clearly shown in Table 1) are mainly due to the number of ports / terminals selected per region, their engagement / importance in the shipping industry, and in a smaller proportion to the lack of local inspectors and logistic arrangements to implement the inspection in some areas.

National Port State ballast water inspections are ongoing and the data this generates are being used to assist Port Captaincies Authorities with cases of non-compliance, and are part of an ongoing research project conducted by the Brazilian Navy's Marine

Research Institute Admiral Paulo Moreira. Furthermore, the ongoing task of enforcement allows the ratification and rectification of adopted procedures and their updates. Currently, the implementation of a uniform procedure of ballast water sampling and analysis along the Brazilian coast is being developed.

Other relevant national ballast water management initiatives were taken in the period, mainly actions taken by Governmental stakeholders, some of them included in the present study with a view to contextualising the proactive way that Brazil is dealing with the ballast water issue. One of the first important ballast water initiatives came from Petrobras, the Brazilian Oil Company, during the 1990's, with the development of the Brazilian Dilution Method proposed as a variation of the flow-through method recommended by the International Maritime Organization (IMO). The method was adopted as one of the three recommended methods to exchange ballast water in mid-ocean. Results from the tests conducted by Petrobras were presented in many IMO papers (MEPC 38/13/2, MEPC 42/8/3, MEPC 42/INF.14, MEPC 53/2/24, MEPC 53/INF.18 and DE 42/11/1) Mauro et al., 2002; Castro, 2008).

The Brazilian Health Surveillance Agency undertook a 6-month campaign in 2001/2002 with a view to investigating the possible presence of pathogenic organisms in ballast waters reaching Brazilian ports. Furthermore, training was given on sampling and analysis of ballast water among local health surveillance agents working in Brazilian ports. More recently, as aforementioned, the Agency updated its regulation on the matter and has started working with other Ministries / National Authorities with a view to combine efforts to have a broader control over the spread of unwanted species along the coast.

In December 2003 the Minister of State for the Environment officially established the Golden Mussel National Task Force which was composed of many entities from Federal, State and Municipal Governments, energy companies like Furnas, Itaipú and



Eletrobrás and was supported by an Expert Group. Through the Golden Mussel National Task Force an Emergency Action Plan was launched, with the involvement of State and local institutions on the control of the golden mussel. The main purpose of the Emergency Action Plan was to control the golden mussel spread in the river basins of Guaíba, Alto Paraguay and Alto Paraná and also in developing outreach activities, by means of public awareness, training and monitoring activities (Castro, 2008). The Brazilian Environment Ministry was the Leader Agency regarding the implementation of the IMO Global Ballast Water Programme (GloBallast) – phase 1 in Brazil, during 2000 and 2004.

Also supported by the Brazilian Environment Ministry, a National Report on Invasive Alien Species within the country was started in mid-2003, with a view to systematizing and disseminating existing information on the subject. Reports on actual or potential invasive species affecting the marine environment, inland waters, human health and agriculture were produced. Results from each subproject were summarized in two main documents: "Diagnosis of Current and Potential Invasive Species" and "Existing Structure for the Prevention and Control". A comprehensive report concerning the marine environment was officially launched in 2009.

During 2007 and 2009, Petrobras undertook research at some of their marine terminals known to be ballast water importers taking into account the cargo loading/unloading rate. The research applied the GloBallast risk assessment methodology in the selected terminals. Results did not show significant risks, except for two shipping routes that had been identified as important paths because the ports of origin had environmental similarity with some of the national terminals studied (personal communications).

### **3.5. Conclusions**

The Brazilian experience shows that very high levels (97%) of compliance with ballast water management regulations can be made to work in a region of global importance to

the maritime industry. Results showed a positive effect of time over the compliance; however, results also showed discrepancies in the inspection regime along the coast. The decrease in non-compliance probably reflects an increase in awareness of the Brazilian Standard and increased industry commitment to minimizing the spread of invasive species.

Since the adoption of ballast water management in Brazil the main goal of the Maritime Authority has been to prevent and minimise impacts associated with the spread of non-native species through ballast water. The Port State Control efforts illustrate Brazilian commitment to marine environment protection and to international laws such as the United Nations Convention on the Law of the Sea, the Convention on Biological Diversity and the Ballast Water Management Convention itself. Moreover it represents, in our view, the best approach to verify the standard's implementation and to comply with IMO recommendations.

Brazil signed the Ballast Water Management Convention on 25<sup>th</sup> January 2005 and adopted its own NORMAM-20 regulations after open discussion within the Brazilian maritime community. Notwithstanding the adoption of a national legislation and the implementation of an inspection regime, the work on the subject is far from over, requiring further scientifically validated data for evaluation of its efficacy, besides monitoring and surveys campaigns to control the spread of non-native species (Lehtiniemi et al., 2015).

Although Brazilian authorities have stated that having international standards are the most effective way to enforce ballast water regulations the pressure to protect the marine environment led to the adoption of unilateral rules. Nevertheless, since the Ballast Water Management Convention only recently reached the combined tonnage of contracting States and came into force on 8 September 2017, the decision taken more than ten years ago appears to have been the right one for biosecurity in Brazilian waters.

## **Chapter 4 : Different approaches and limitations for testing phytoplankton viability in natural assemblies and treated ballast water**

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## **Abstract**

Shipping is recognised as an unintentional efficient pathway for spreading aquatic non-native species. In 2004, a unique IMO Convention was adopted to control and minimise this transfer through ships ballast water. This BWM Convention entered into force on 8<sup>th</sup> September, 2017. However, unlikely the majority of IMO Conventions, the Ballast Water Management Convention requires ships to comply with biological standards (e.g. concentration of organisms per unit of volume in ballast water discharges). This study aimed to apply different techniques developed to measure concentrations of phytoplankton in natural and treated ballast water samples and compare them with the established flow cytometry method and vital staining microscopy. Samples were collected in the English Channel over one year and on-board during ballast water shipboard efficacy tests. 23% to 89% of the total of phytoplankton were viable, whilst for cells larger than 10 µm (a size defined by the BWM Convention) the percentage varied from 3% to 60%. An overall good correlation was seen between the measurements taken with the two fluorometers and in comparison with the flow cytometry analysis, as found in previous studies. Analysis of treated ballast water samples showed a large variation in the number of viable cells, however indicating the same level of risk on all occasions for regulatory purposes. One of the key aspects to bear in mind when sampling and analysing for compliance is to be aware of the limitations of each technique.

Keywords: phytoplankton, chlorophyll a, fluorescence, compliance, shipping.

## **4.1. Introduction**

### **4.1.1. Chlorophyll fluorescence**

Chlorophyll fluorescence is used as a non-destructive tool for studying phytoplankton for investigating photosynthesis (Genty et al, 1989; Govindjee, 2004). Chlorophyll a is

present in all photosynthesizing plants and algae, which highlights its unique characteristics (Guilbault et al., 1990). Fluorescence occurs when a photon is absorbed and an electron is transported to an excited, unstable state of energy. The electron subsequently returns to the non-excited state resulting in the emission of electromagnetic radiation. In photoautotrophic organisms this process is only possible because of photosystems consisting of unique protein complexes in chloroplasts that are responsible for light absorption and essential for photosynthesis. There are two photosystems in photoautotrophic organisms, photosystems I and II (known as PSI and PSII). PSII is where oxygen is released as a by-product and PSI is where carbohydrates are formed.

The principle underlying analysis using chlorophyll fluorescence is relatively simple: when light is absorbed by chlorophyll in cells it can be used to drive photosynthesis, it can be dissipated as heat or it can be reemitted as chlorophyll fluorescence (Bradbury & Evennett, 1996; Maxwell & Johnson, 2000). From the perspective of photosynthetic organisms, fluorescence represents a waste of energy; however the amount is quite low with a maximum of circa of 3% of the absorbed light (Guilbault et al., 1990).

Marine ecosystems comprise only about 1% of Earth's photosynthetic biomass, yet are responsible for about 50% of our planet's annual net primary production (Geider et al., 2001; Falkowski et al., 2004). Photosynthetic activity in the oceans comes from a very diverse range of organisms including phytoplankton, macroalgae, and symbiotic invertebrates (Falkowski et al., 2004).

#### **4.1.2. Measuring phytoplankton activity**

Due to the fact that it is non-destructive, expeditious and precise, chlorophyll fluorescence has become a routine technique for studying biomass as well as the photosynthetic activity of photoautotrophic organisms (Govindjee, 1995; Govindjee, 2004). Many techniques have been developed with a view to using chlorophyll

fluorescence as a measure of photosynthetic primary production and photochemical efficiency. 1Hz Fluorometers, Pulse-Amplitude Modulated Fluorometers (PAM), Dual-Modulation LED Kinetic Fluorometers and the fast repetition rate Fluorometers (FRRF) are among the tools used to measure chlorophyll fluorescence (Kolber et al., 1995; Schreiber 1998, Wilhelm, 2003). Principles employed in the different techniques basically differ in how the photochemistry is saturated to generate the maximum fluorescence yield ( $F_m$ ) (Röttgers, 2007). In addition to the dark-state (i.e. the state of a, in this case, dark-adapted molecule that cannot absorb (or emit) photons) ground fluorescence (known as  $F_0$ ), maximum fluorescence ( $F_m$ ) and consequently variable fluorescence ( $F_v$ ) can be measured ( $F_v = F_m - F_0$ ) and is often used as an indicator of the vitality of the phytoplankton.

Techniques using stains that can penetrate and bind to cell DNA have been developed that allow the investigation of viability of cells in the marine environment (Agustí & Sanchez, 2002). These stains have also been applied to the measurement of cell viability in the field of ballast water. Stains that fluoresce yellow / green under excitation by certain wavelengths of light have been generally adopted or proposed because they do not interfere with the red fluorescence of the chlorophyll (Veldhuis et al.1997; Tang & Dobbs, 2007). The ability to measure the viability of phytoplankton cells helps, for instance, in distinguishing living cells in the water column from non-functional cells that are still capable of fluorescing but contribute to over estimation of viable chlorophyll-a biomass (Veldhuis et al., 2001; Agustí & Sanchez, 2002; Llewellyn et al., 2005; Steele, 2014). In that sense, previous studies have detected a large number of dead cells in the water column (ca. 95%) at certain periods of the year (Veldhuis et al., 2001; Agustí & Sanchez, 2002), highlighting the importance of discriminating viable from non-viable cells particularly when determining regulatory compliance.

#### **4.1.3. Fluorometry**

Two different fluorometers were used in this study to measure the chlorophyll fluorescence of phytoplankton groups and determine their viability. The differences between the two are mainly related to the way the light pulse saturates the Photosystem II Reaction Centre and, as a result, different fluorescence values are detected. The basic concepts are explained briefly hereafter (sections 4.1.3.1 and 4.1.3.2).

##### **4.1.3.1. Multiple turnover**

The technique delivers a series of modulated pulses of milliseconds to induce fluorescence excitation ( $F_0$ ) followed by a set of saturating light pulses to measure the maximum fluorescence ( $F_m$ ), using light of different intensities to determine the photosynthetic efficiency (Schreiber, 1998). The modulation of the fluorescence is needed so the actinic light and the fluorescence itself can be distinguished (Schreiber, 2004). Taking into account that fluorescence and photochemical energy conversion are competitors in the process, two extreme situations might occur: when all the reaction centers in the photosystem are open (samples in a dark-adapted state) and when they are all closed due to the saturation light; which leads to  $F_0$  and  $F_m$  measurements respectively.

One distinguished feature of multiple turnover systems is that increased  $F_v/F_m$  values are found, usually between 15 and 20% but can be up to 28% higher than the ratio measured with single turnover systems (Kolber et al.; 1998; Samson et al.; 1999; Röttgers, 2007). The increased values are caused by an increased  $F_m$  resulting from longer saturation pulses able to reduce the primary electron acceptors and possibly the secondary acceptors and plastoquinone, all involved in the electron transport chain (Kromkamp & Foster, 2003; Röttgers, 2007).

For the purposes of the present study, samples were analysed with the Ballast Check 2 a pulse-amplitude modulated fluorometer, from Turner Designs, which provides a quick estimation of number of cells for the fraction larger than 10  $\mu\text{m}$  and the photosynthetic activity in the sample.

#### **4.1.3.2. *Single turnover***

In single turnover systems the saturation pulse is sufficiently bright to saturate the photosystem II and to lead to a single reduction of all primary electron acceptors. This high-energy flash is then enough to increase the ground fluorescence ( $F_0$ ) to a maximum ( $F_m$ ) (Kromkamp & Foster, 2003). Single turnover pulses of microseconds are also short enough to allow a rapid recovery between successive pulses.

In this study we use the FastBallast single turnover fluorometer, from Chelsea Technologies Group, that uses a combination of four LED channels with emission spectra centred at 450, 470, 530 and 624 nm. Default values adopted by the instrument (royal and standard blue) are able of maximize the chlorophyll b and c fluorescence signals due to the fact that these two chlorophylls are the main accessories light-harvesting pigments for cells between 10 and 50  $\mu\text{m}$  (phytoplankton size class addressed by the BWM Convention) (Oxborough, 2016). Notwithstanding the fact that cells smaller than 10  $\mu\text{m}$  can also contain these pigments as discussed in the FaB handbook (Oxborough, 2018). The array of LED combination therefore provides the possibility of detection of a wide range of phytoplankton groups (diatoms, dinoflagellates, green algae, cyanobacteria and coccolithophores).

#### **4.1.4. Ballast water regulations**

The International Maritime Organization's Ballast Water Management Convention (IMO BWMC) entered into force on 8<sup>th</sup> September, 2017, after a delay of more than 13 years from its adoption on 13<sup>th</sup> February, 2004. The Treaty was preceded by two sets of guidelines developed during the 1990s whilst progressing its work towards the



development of an international convention; The International Guidelines for Preventing the Introduction of Unwanted Aquatic Organisms and Pathogens from Ballast Water and Sediment Discharges (resolution MEPC.50(31) in 1991 (subsequently adopted as the IMO Assembly resolution A.774(18) in 1993) and the IMO Assembly resolution A.868(20) - Guidelines for the Control and Management of Ships Ballast Water to Minimize the Transfer of Harmful Aquatic Organisms and Pathogens (1997).

Also during the 1990s a landmark step was taken, with recognition by the United Nations (UN) Conference on Environment and Development, on the ballast water issue as a major international concern. With the adoption of the Convention on Biological Diversity by the UN (Rio 92) the threat represented by the transfer of non-native species was explicitly identified as one of the four greatest threats to the world's oceans.

Apart from phytoplankton species, there are many emblematic examples of invasive species recorded during the 1980s and early 1990s around the globe e.g. the golden mussel (*Limnoperna fortunei*) in South America (Darrigran & Pastorino 1995), the zebra mussel (*Dreissena polymorpha*) in North America (Hebert et al.; 1989) and the comb jelly (*Mnemiopsis leidyi*) in Europe (Kideys, 1994). Within the Ballast Water Management Convention, a ballast water performance standard known as the D-2 standard defines maximum allowable concentrations of organisms in the discharged ballast water according to their size or group (Table 4-1); where the lower size range consists mainly of phytoplankton. Unilateral regulations have also been adopted in some countries (e.g. Standards for Living Organisms in Ships Ballast Water Discharged in U.S. Waters, 2012, United States Coast Guard (USCG)) with similar requirements.

**Table 4-1. IMO's Ballast Water Management Convention regulation D-2 (IMO, 2004)**

Organisms/Indicators and size class	Maximum allowable number in discharged water according to the Regulation (CFU <sup>1</sup> = Colony Forming Unit)
Viable organisms $\geq 50 \mu\text{m}$ in minimum dimensions	less than 10/m <sup>3</sup>
Viable organisms $\geq 10 < 50 \mu\text{m}$ in minimum dimensions	less than 10/mL
Toxicogenic <i>Vibrio cholerae</i> (O1 and O139)	less than 1 CFU/100 mL
<i>Escherichia coli</i>	less than 250 CFU/100 mL
Intestinal Enterococci	less than 100 CFU/100 mL

To meet the requirement for minimising the numbers of viable organisms within ballast water tanks, a variety of ballast water management systems (BWMS) have been developed which are mainly based on an initial filtration step plus a chemical or physical treatment. Electrochlorination and treatment using ultra-violet (UV) irradiation are the two main secondary treatments. Both treatments have pros and cons and their use needs to be evaluated together with the ship type, trading route and environmental aspects.

UV-C systems are often recommended as environmentally friendly systems as no potentially toxic by-products are release to the environment during the discharge

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<sup>1</sup> In microbiology, the Colony Forming Unit (CFU) is a unit of measure used to estimate the number of cells capable of multiplying under controlled conditions, i.e. the number of viable cells in the sample.

(Batista et al., 2017). The main disadvantage however is related to the regrowth of many species of phytoplankton after a period varying from six to twelve days regardless the UV-C radiation dose (Martínez et al., 2012; Martínez et al., 2013; Stehouwer et al., 2015). In addition, UV-C systems have lower biological efficacy in high turbidity waters because UV light transmission is considerably reduced. Finally, there is a 'delayed kill effect' on organisms (Werschkun et al., 2014; First and Drake, 2014; Stehouwer et al., 2015).

Electrochlorination based ballast water treatment relies on the process of producing hypochlorite (a powerful oxidant) when an electric current is run through water containing a minimum concentration of salt. Yet electrochlorination is usually more efficient when used in waters of high turbidity (Batista et al., 2017). In contrast to UV-C irradiation systems, the hypochlorite generated in these systems may need to be neutralized before discharge and the dose is applied just once during the treatment (while UV-C treatment takes place during water uptake and discharge). Other concerns are related to the influence of lower temperatures on a system's efficacy and on the acceleration of tank corrosion (Morris, 1966; Lysogorski et al., 2011).

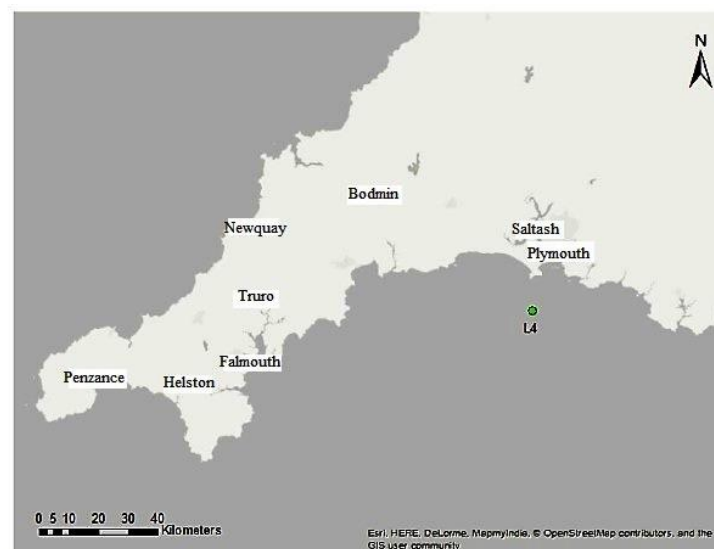
Considering the paramount importance of fluorescence applications on viability studies and for ballast water compliance issues, this study examines the use of different fluorescence techniques to measure viability and abundance of phytoplankton, the dominant group in the IMO D-2 size range 10-50  $\mu\text{m}$  (Table 4-1). The pattern of distribution of viable and non-viable cells was investigated over one year in a natural assembly using a flowcytometer as well as two fluorometers (with different excitation techniques) measuring the chlorophyll *a* biomass and the number of cells. Likewise, ballast water samples from commercial efficacy testing were also measured with both fluorometers and the results compared with those from flow cytometry and epifluorescence microscopy analysis using stains. The primary objective was to identify patterns on the phytoplankton size distribution with regards to the viability of cells in a

natural assembly and possible benefits and limitations of the techniques in the context of the ballast water compliance issues.

## 4.2. Material and Methods

### 4.2.1. Area of study

Station L4 of the Western Channel Observatory (WCO), located in the English Channel, about 13 km from the coast (coordinates 50°15.0'N; 4°13.0'W) (Figure 4.1), was used to investigate natural phytoplankton assemblages (see Castro & Veldhuis, 2018 for details). The WCO is well characterised through ongoing research projects conducted by the Plymouth Marine Laboratory (PML) and the Marine Biological Association (MBA). The L4 area is known to be influenced by inputs of nutrients from rivers together with oceanic influences (Pingree and Griffiths, 1978; Woodward et al., 2017). Weekly samples were collected from the surface with a bucket between June 2016 and July 2017.



**Figure 4.1.** L4 sampling site in the English Channel (50°15.0'N; 4°13.0'W).

#### **4.2.2. Ballast Water Shipboard Biological Efficacy Tests**

Eight on-board tests of a commercial ballast water treatment system were conducted between 2016 and 2017. The Ballast Water Treatment System (BWTS) used was certified and based on UV-C disinfection. On all occasions, sampling occurred during the discharge of ballast water while in port.

Samples were taken from the sampling point in the discharge line provided in each ship (Figure 4.2). A sterile sampling tube was fitted directly to the sampling valve on the BWTS. The ballast water discharge was run for 5 minutes prior to the first sample (to avoid debris in the ballast water lines). Ideally six samples are taken over typically 1 hour of discharge from a single tank or the simultaneous discharge of two tanks. The number of samples varied among tests from two to six (plus replicates) due to pumping rates and varying volumes of water in each tank.



**Figure 4.2. Sampling arrangements during ballast water shipboard tests.**

#### **4.2.3. Methods**

##### ***4.2.3.1. Flow cytometry***

Flow cytometry (FCM) allows the analysis of a variety of properties related to size (Forward Light Scatter: FS) and optical density (Side Scatter: SS) of auto or induced

fluorescence generated by individual cells and is widely applied in biological research including phytoplankton (Davey & Kell, 1996, Legendre et al., 2001; Ormerod, 2009).

All L4 samples after collection were immediately returned to PML, where they were analysed at the flow cytometry facility using a Bekton Dickinson FACSort™ flow cytometer. Samples were analysed for five minutes in a high flow rate of approximately 225  $\mu\text{L min}^{-1}$  (total volume 1.125 mL), as follows:

- 2 mL living samples;
- 2 mL living stained samples;
- 2 mL dead samples; and
- 2 mL dead stained samples.

Flow rates were calibrated with Beckman Coulter Flowset fluorospheres of a known size and concentration. SYTOX Green dye was used as a nucleic acid stain for live/dead determinations (See section 2.3.3). Samples were also killed by heating at 80°C for five minutes in a water bath before analysis followed by stained analysis as described. FCM data were analysed with the FCS Express Flow Cytometry Software, version 5 (Denovo Software).

FCM settings were set to display cells in the size range from 2 to 50  $\mu\text{m}$ . The size was measured as the scattered light in the forward direction (FS), the measurement best related to size (Ormerod, 2009, Castro & Veldhuis, 2018). The red fluorescence from the phytoplankton chlorophyll a pigment (emission > 630 nm) was measured after excitation with blue laser light (488 nm) while stained samples fluoresced bright green (emission peak of 523 nm). Standard spherical beads with known diameters (9.7 and 50  $\mu\text{m}$ , Polysciences) were used as an internal standard for instrument calibration. These beads are uniform in size with known coefficients of variation (C.V. <2%) and measurements should possess the same spread for size and fluorescence.

Data analysis was based on clustering (sub) populations with identical size and chlorophyll fluorescence properties. The total number of phytoplankton cells (total number of cells/mL) was derived from the analysis of stained living samples (living + dead cells) while viable phytoplankton cells were identified by the red fluorescence of living samples.

#### **4.2.3.2. Fluorometry**

The Ballast Check 2 (BC2) procedure includes a filtration step (using a 10 µm mesh filter) to estimate the abundance of cells >10 µm based on the conversion of a fluorescence value divided by a fixed constant value of chlorophyll fluorescence per cell for the size range of 10 – 50 µm. In a separate run the total chlorophyll fluorescence of the sample was measured using a syringe filter of 0.2 µm mesh. Default results displayed on the screen of the equipment are the abundance of cells in the sample as well as the photochemical efficiency ( $F_v/F_m$ , a measure of the effects of stress/vitality on the cell). The BC2, using default settings, provides a risk indication with regards to the IMO D-2 ballast water performance standard: high or low, depending on the combination of the abundance (no of cells/mL) and photosynthetic activity. According to the equipment manual, high risk water samples give an abundance > 10 cells per mL and a  $F_v/F_m > 0.25$ . When the number of cells is < 10 cells per mL or  $F_v/F_m < 0.25$ , then the equipment displays a low risk indication (Table 4-2). Another important aspect is that the photosynthetic activity ( $F_v/F_m$ ) is reported as not-detected (ND) whenever its value is outside the range 0.01 to 0.75. The upper detection limit of the instrument is > 2,000 cells per mL and, when seen, a high risk is displayed in the screen.

**Table 4-2. Ballast Check 2 risk assessment readings and advised action as recommended in the user manual (Ballast Check 2 User Manual – Rev.1, 5th Sept 2016).**

Readings Risk	<ul style="list-style-type: none"> <li>• Abundance (cells/mL)</li> <li>• Activity (Fv/Fm)</li> </ul>	<ul style="list-style-type: none"> <li>• Interpretation</li> <li>• Advised action</li> </ul>
LOW	$< 10$ $< 0.25$	Within D2 Guidelines Maintain BWTS performance
LOW	$< 10$ $\geq 0.25$	Within D2 Guidelines Maintain BWTS performance
LOW	$> 10$ $< 0.25$	Within D2 Guidelines Maintain BWTS performance
HIGH	$> 10$ $\geq 0.25$	Exceeds D2 Guidelines Retest from sample flow. Check BWTS performance. If results remain high, plan for a more detailed analysis at earliest opportunity.

In this study the equipment was connected to a laptop during analysis allowing the reading of all fluorescence parameters being measured ( $F_0$ ,  $F_m$  and  $F_v/F_m$ ) through the HyperTerminal software (Hilgraeve, Inc).

The FaB fluorometer (Chelsea) has two analysis steps. The initial level (Level 1) provides a numeric value that relates to cell density (usually equals to  $F_v \times 1000$  or  $F_v \times 100$  depending on the software version) where  $< 0.04$  indicates a “pass” and a numeric value  $> 40$  indicates a “fail”. Whenever the sample produces results between these two values, the system will continue to a Level 2, where cell density is estimated from the distribution of  $F_v$  values within several hundred semi-discrete measurements, alternatively to the amplitude of  $F_v$  derived from a single measurement (Oxborough, 2018). After about six minutes (in addition to the two minutes for level 1 analysis) the actual cell density in the sample is displayed. The software FaBtest gives the user different possibilities for obtaining further information during the data acquisition and analysis. For this study, samples were measured with and without filtration giving total



cells in the range of 2 – 50 µm and, using a 10 µm mesh filter and subtracting the results from the total, numbers of cells between 10 and 50 µm.

All samples were kept in dark (i.e. dark adapted) for at least fifteen minutes before analysis.

#### **4.2.3.3. Vitality staining**

To test the viability of phytoplankton cells, the nucleic acid specific stain SYTOX Green™ (S-7020; Molecular Probes, Inc.) was used to indicate cells with compromised membranes since this dye can only penetrate such cells which then fluoresce bright green when excited (Roth et al., 1997; Veldhuis et al., 2001). The SYTOX Green is available in a 5mM solution and that requires a 100x dilution before use. Working stocks were prepared by diluting 50 µL in 5 mL of ultrapure water (Mili-Q water). For flow cytometric analysis, 2 mL samples of seawater were mixed with 20 µL of the SYTOX Green working stock and kept in the dark for a minimum of 15 minutes prior analysis. As described by Veldhuis et al. (2001), cells exposed to the dye which stained bright green were classified as dead cells and together with the non-stained (viable) cells that exhibited red emission fluorescence were considered the total phytoplankton community in the sample.

Another fluorescent staining method used in this study for ballast water samples was the one recommended by the IMO and USCG for detecting viable cells in the  $< 50 \geq 10$  µm size group. This method uses a combination of two vital stains: Fluorescein Diacetate - FDA (Molecular Probes-Invitrogen) and 5chloromethylfluorescein diacetate – CMFDA (CellTracker™ Green; Molecular Probes-Invitrogen) (Steinberg, 2011). In contrast to SYTOX Green which is a dead-stain, FDA is a live-stain. All BWTS tested in this study were UV-C disinfection technologies, therefore, samples were stored for 24 hours at ambient seawater temperature in the dark before analysis, in order to provide enough time for the UV-C damage to take effect.

#### 4.2.4. Statistical data analysis

All statistical analyses were carried out using IBM SPSS Statistics software (Version 23 and 24), Microsoft Excel (Analysis ToolPak) and Primer 7 (version 7.0.13) from Primer-e (Quest Research Limited).

### 4.3. Results

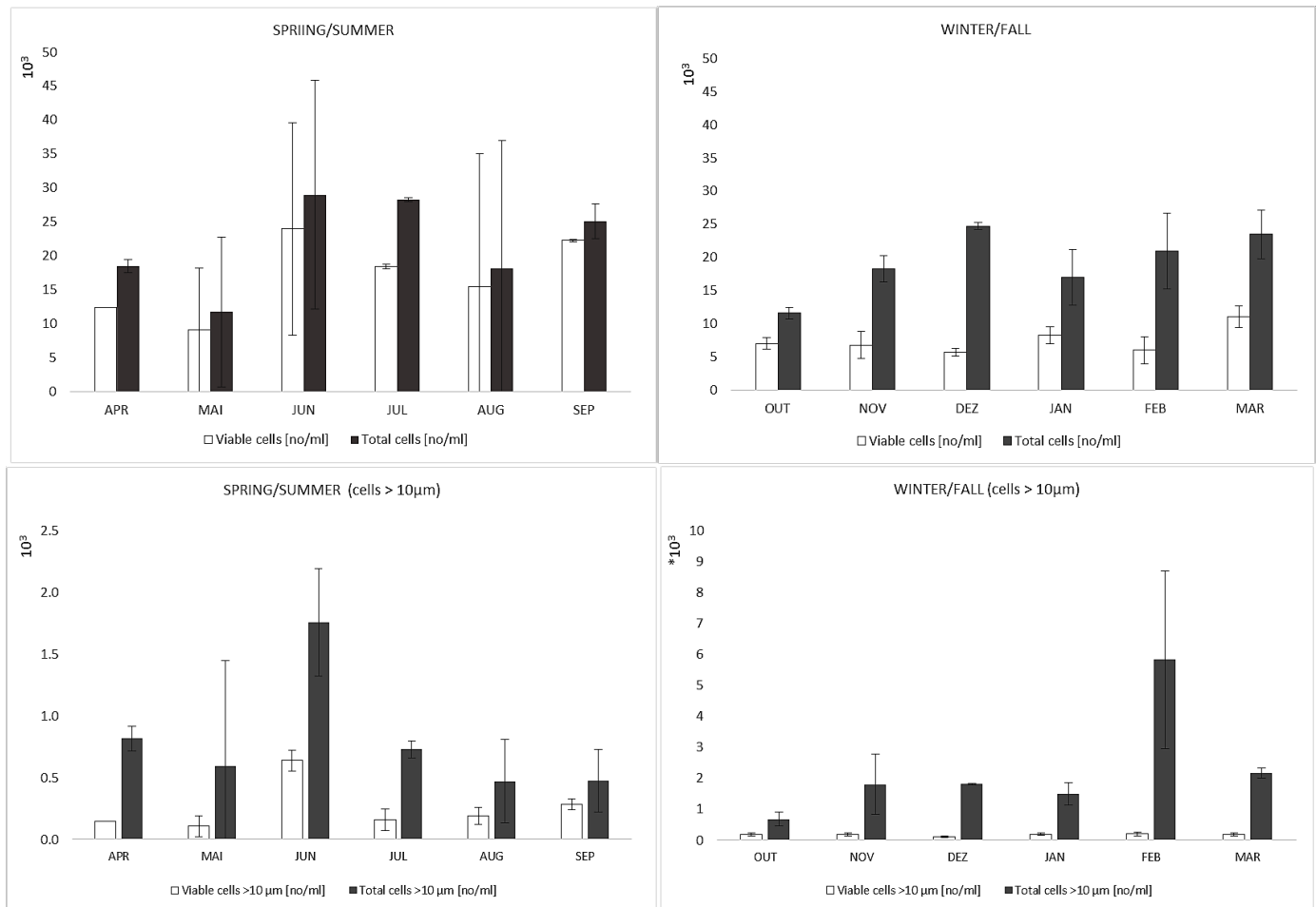
#### 4.3.1. Annual field data

L4 samples collected at the surface from June 2016 until July 2017 showed an averaged abundance of 20,153 cells/mL from which 13,179 in average were viable cells (no/mL) in the size range of 2 to 50  $\mu\text{m}$ . Within this cell size class, the majority of cells detected was between 2 and 10  $\mu\text{m}$  of size (ca. 98%) (Table 4-3).

**Table 4-31. Mean number of total and viable cells (no/mL) at surface of the water column considering cells between 2 and 50  $\mu\text{m}$  and the fractions between 2 - 10  $\mu\text{m}$  and >10 - 50  $\mu\text{m}$ . Samples were collected from June 16 to July 17 at L4 sampling site, in the English Channel.**

Size class	Mean (Total cells $\pm$ SD)	Mean (Viable cells $\pm$ SD)
Total cells 2 – 50 $\mu\text{m}$ (no/mL)	20153 $\pm$ 11718.9	13179 $\pm$ 11401.2
>2 - < 10 $\mu\text{m}$ (no/mL)	15974 $\pm$ 9558.3	10392 $\pm$ 9659.5
> 10 $\mu\text{m}$ (no/mL)	1404 $\pm$ 1575.4	203 $\pm$ 171.9

During the winter at L4 (October to March) the lowest numbers of living cells were found (23%;  $CV \pm 10\%$ ) at surface. On the other hand, the highest values were found during the summer period at L4 (spring + summer) with a peak of 89% in September ( $CV \pm 31\%$ ). For cells  $> 10 \mu m$ , abundance of viable cells dropped from 60% in September to 3% in February ( $M= 26.9$ ;  $CV \pm 96.2\%$ ) (Figure 4.3).

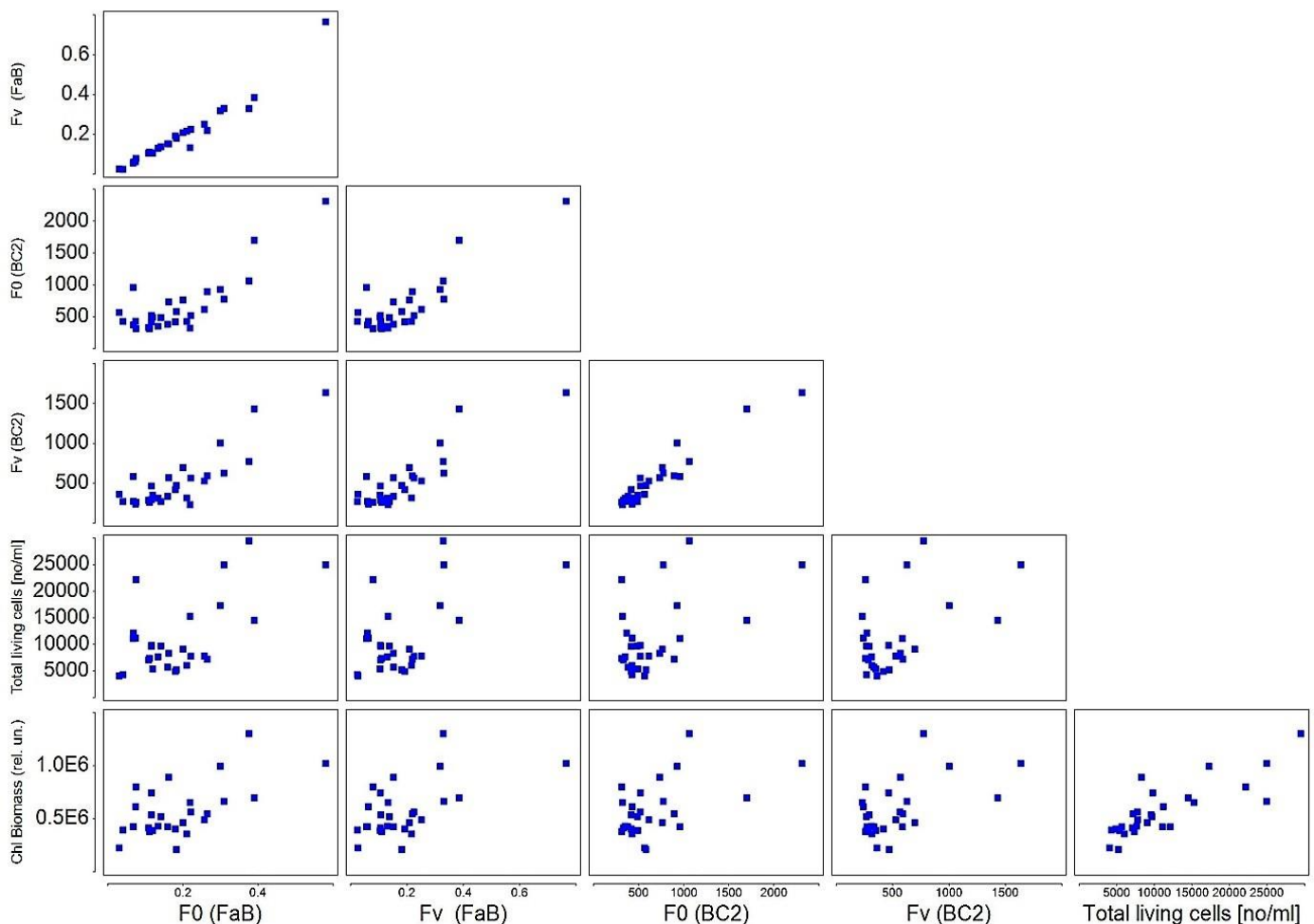


**Figure 4.3. Number of viable and total cells (no/mL) in the size range 2 to 50  $\mu m$  and  $> 10$  to 50  $\mu m$  according to the season at L4. Samples collected at the surface at L4 sampling site from June 2016 to July 2017.**

Throughout the entire sampling period, fluorometers were available for analysis in conjunction with the FCM, from August/2016 to April/2017 uninterruptedly. A Draftsman plot (Figure 4.4) and its correlations coefficients (Table 4-4) are presented to determine the covariation between the chlorophyll parameters  $F_0$  and  $F_v$  measured with both

fluorometers and the abundance of cells and the chlorophyll biomass (abundance of cells \* red fluorescence) based on flow cytometrical measurements (cf Castro & Veldhuis, 2018). The latter was done to determine the variation in cellular chlorophyll concentration due to changes in cell size, since a co-variation is expected as demonstrated by Castro & Veldhuis, 2018.

Results from the correlation coefficient between  $F_0$  and  $F_v$  measured using the two fluorometers showed a strong correlation ( $> 0.8$ ); when compared to the number of cells/mL and the chlorophyll biomass detected with the FCM, results obtained with the fluorometers showed a moderate / relatively strong covariation (Table 4-5).



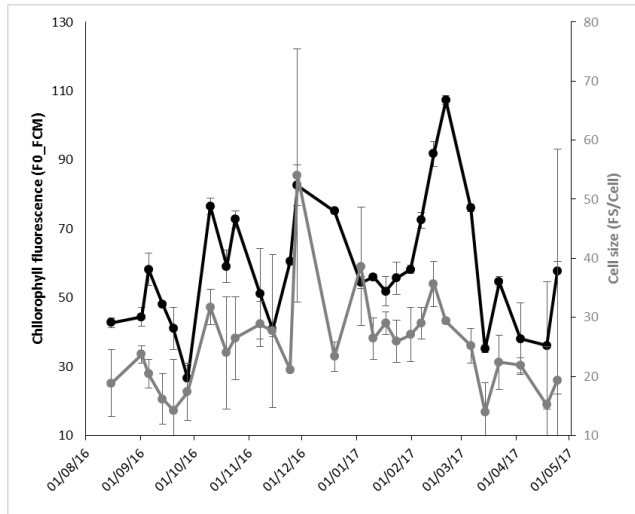
**Figure 4.4. Covariation between chlorophyll fluorescence ( $F_0$  FaB and  $F_0$  BC2) and variable fluorescence ( $F_v$  FaB and  $F_v$  BC2) measured with the two fluorometers and the number of total living cells between 2 and 50  $\mu\text{m}$  [no/mL] and chlorophyll biomass detected with the FCM (Chl biomass). L4 surface samples collected from August/2016 to April/2017.**

**Table 4-4. Correlations coefficients among F<sub>0</sub> and F<sub>v</sub> measured with the two fluorometers and the number of living cells ([no/mL]) and chlorophyll biomass measured with the FCM (Chl biomass). L4 surface samples collected from August/2016 to April/2017.**

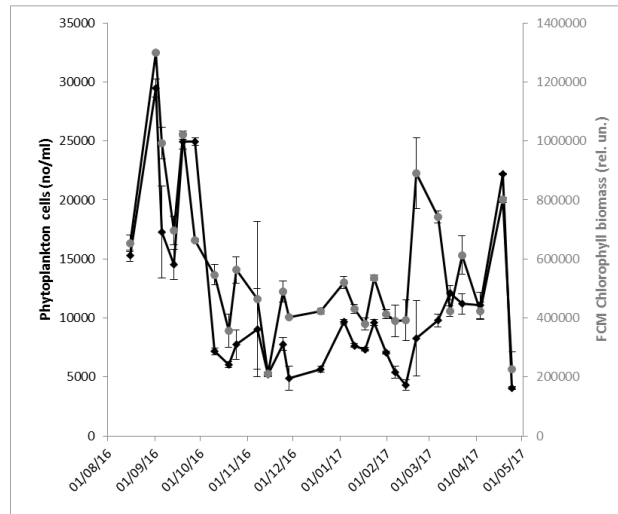
	F <sub>0</sub> (FaB)	F <sub>v</sub> (FaB)	F <sub>0</sub> (BC2)	F <sub>v</sub> (BC2)	Living cells[no/mL]
F <sub>0</sub> (FaB)					
F <sub>v</sub> (FaB)	0.97				
F <sub>0</sub> (BC2)	0.82	0.86			
F <sub>v</sub> (BC2)	0.85	0.88	0.96		
Living cells [no/mL]	0.61	0.58	0.52	0.51	
FCM Chl biomass	0.62	0.56	0.53	0.56	0.81

The estimated number of cells provided by the two fluorometers was based on the amount of chlorophyll detected in each living cell (using a fixed value per cell set within the instrument firmware). Therefore, a good correlation between the chlorophyll fluorescence measurements obtained with the two fluorometers and the chlorophyll biomass obtained with the flow cytometer is expected.

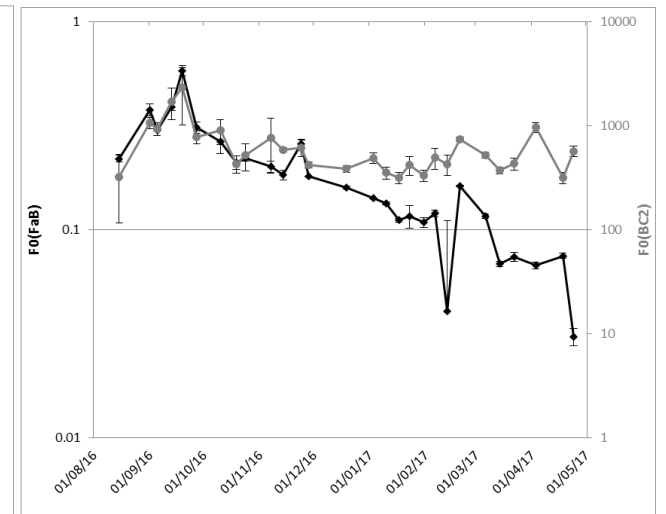
Chlorophyll content, cell size and the number of viable cells were investigated during the sampling period. The lower number of viable cells and therefore lower chlorophyll biomass measured with the FCM was found in the winter (Figures 4.3 and 4.5b). The lower number of cells was accompanied by bigger cells and therefore by higher chlorophyll a contents per cell measured with the FCM (Figure 4.5a). F<sub>0</sub> measured with the fluorometers in general were more sensitive to the trend observed for the number of cells in the period of sampling with higher chlorophyll fluorescence values obtained for the summer while lower values were seen in the winter (Figure 4.5c).



(a)



(b)

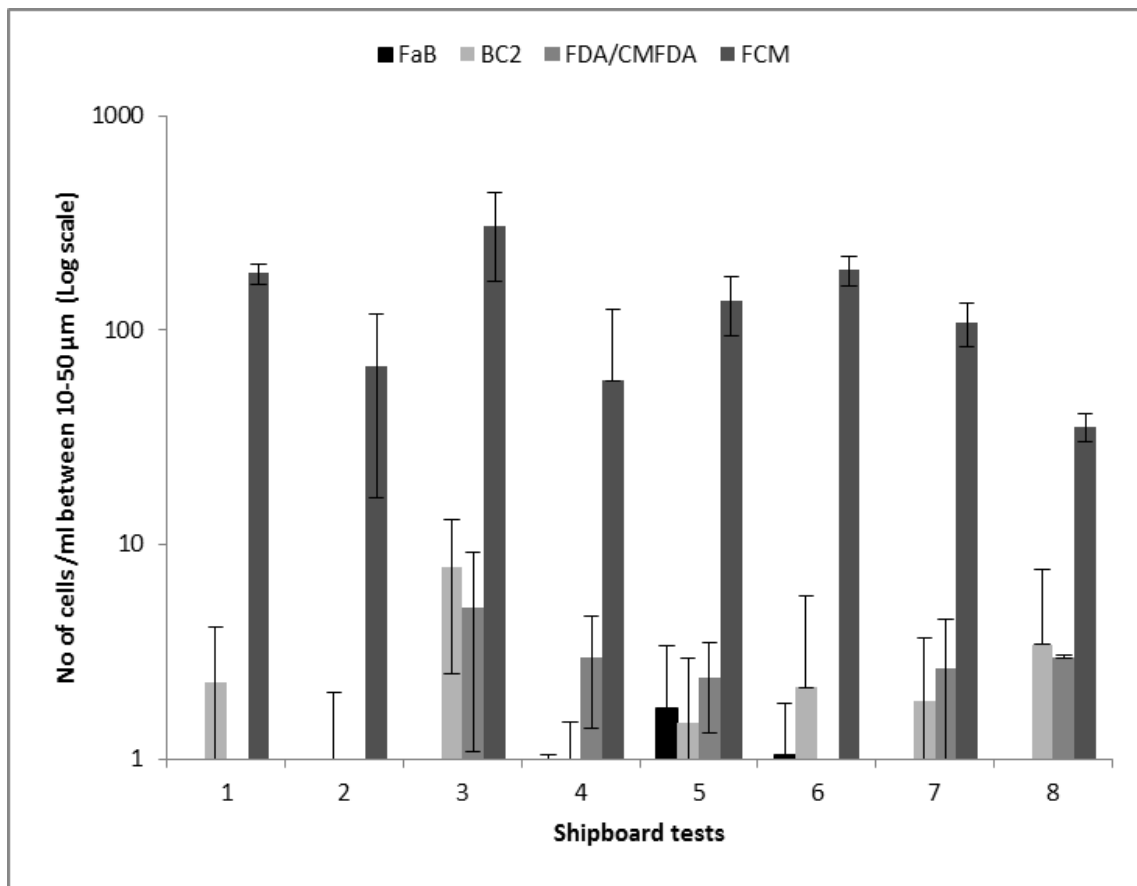


(c)

**Figure 4.5. a) chlorophyll fluorescence (F0\_FCM) and cell size distribution (FS/cell) measured with the flow cytometer; (b) number of viable cells (no/mL) and the derived flowcytometric chlorophyll biomass (no of cells \* red fluorescence); and (c) chlorophyll biomass measured with the fluorimeters (F<sub>0</sub>(FaB) and F<sub>0</sub>(BC2)). Samples collected at the surface at L4 sampling site, from August 2016 to April 2017.**

#### 4.3.2. Ship-board data

Eight biological efficacy tests of BWM systems on board of ships were conducted between 2016 and 2017. Samples taken were analysed for the abundance of phytoplankton cells in the range between  $\geq 10 - < 50 \mu\text{m}$  to ensure compliance with international regulations in place (e.g. IMO, 2004, USCG, 2012). Results obtained using the two fluorometers together with epifluorescence microscopy (FDA/CMFDA staining) were in all cases very different from results obtained with the flowcytomerically collected data, except on one occasion. Despite the small number of subsamples, co-variance analysis was conducted taking into account the results obtained with the two fluorometers and the epifluorescence microscopy; the latter considered the most accurate of the three. No linearity was observed between the abundance results found with the portable instruments and the number of cells counted using the epi-fluorescence microscopy vital staining assay. In addition, a very large variation between replicates was measured (Figure 4.6). Comparing F0 results obtained with the fluorometers and the FCM, significant results with a relatively moderate strength were found between FaB(F0) and FCM Chlorophyll biomass for the total population and for the fraction  $> 10 \mu\text{m}$  ( $r_s = 0.42$ ).



**Figure 4.6.** Abundance results (no of cells/mL) obtained with the FaB, BC2, FDA/CMFDA assays and FCM (number of cells/mL) for eight shipboard tests conducted in 2016/2017 for cells equal or larger than 10 and smaller than 50  $\mu\text{m}$ .

#### 4.4. Discussion

Viability results from samples collected at L4 over a whole year showed the dominance of viable phytoplankton cells for most of the time except for during the winter period (October to March) (Figure 4.4). The percentage of living cells with a cell size  $> 10 \mu\text{m}$  of the total number of living cells throughout the year was low but consistent (3% in average) and followed the trend observed for total living cells with higher numbers during the summer. However, values were always  $\leq$  than 40% of the total in the size



class except for a peak of 60% in September ( $CV \pm 29\%$ ). Results from the correlation coefficient between  $F_0$  and  $F_v$  measured with the two fluorometers showed a strong correlation (0.82 and 0.88 respectively), confirming earlier results using different types of fluorometers (Gollasch et al., 2012; Bradie, 2016). Compared to FCM results, moderate / relatively strong correlation coefficients were found (around  $r_s = 0.5$  for  $F_0BC2$  and  $r_s = 0.6$  for  $F_0FaB$ ) (Fig 4.4; Table 4-4).

Depending on the time of the year, a higher contribution of dead cells can be found among the marine phytoplankton populations as well as a fluctuating amount of chlorophyll *a* due to environmental changes (Veldhuis and Kraay, 2000; Veldhuis et al., 2001). In this study, abundances of living cells in the water varied from 23% (December) to 89% (September) of the total, whilst for cells larger than 10  $\mu m$  the percentage varied from 3% (February) to 60% in September (Figure 4.3). However, a lower number of cells/mL covaried with a larger cell size and higher chlorophyll *a* (Figure 4.5). Cell numbers determined with both fluorometers are based on the amount of variable fluorescence, i.e. active fluorescence. A lower number of viable cells would therefore result in a lower  $F_v$  and subsequently in a lower calculated cell number using the conversion factor. In contrast the flow cytometer measures a fixed fluorescence only varying with cell size. As a result, the flowcytometric numbers of total cells would over-estimate the actual number of viable cells. Therefore, fluctuations in the numbers of viable cells during the year might be expected to show up different responses according to the technique used and possibly strong deviations among replicates, certainly in the samples with a low number of cells.

Analysis of biological efficacy of ballast water samples applying different methods showed varying results, largely differing from the FCM results. This implies that for the present disinfection technology (UV-C) the remaining phytoplankton cells are

dominated by intact but dead cells. In this regard, for low concentrations of living cells an adequate calibration of the equipment is crucial considering all the other debris and contaminants that are to be expected in ballast water samples. This may be done by analysing, for instance, samples with known concentrations. Romero-Martínez et al. (2017) using a FlowCAM for blank samples of Milli-Q water detected ca. 100 contaminants / mL, enough to compromise the analysis of samples where low concentrations of living cells are expected (e.g. treated ballast water samples). Inorganic mineral particles, for instance, may be a potential source affecting the counting of non-viable cells. Significant relationships ( $p$ -values  $< 0.001$ ) of relatively moderate strength (ca.  $r_s = 0.42$ ) were observed between  $F_0FaB$  and FCM chlorophyll biomass.

Results for number of cells provided by the two instruments and counted on the microscope differed from one another and neither linear relationship nor significant correlation could be observed (Figure 4.6). The large variability may be seen as a sign that the detection methods are far from perfect. However, it should be noted that the fluorescence generated with the vital staining (from enzymatic activity) and the variable fluorescence of cells measured with the other techniques are not necessarily expected to co-vary or to be strictly correlated (Danish Environmental Protection Agency, 2017). Another important aspect is the fact that the use of these stains do not necessarily work as assumed for some microorganisms including phytoplankton, depending on how stains interact with the target organisms (Hammes et al., 2010; Cullen & MacIntyre, 2016; Blatchley et al., 2018).

Alliance for Coastal Technologies reports using field trials, showed that the linear relationship between abundance and concentration of organisms can vary significantly due to the interplay of the environment. As a result the coefficient of determination is

not necessarily highly correlated to the measured concentration of organisms  $\geq 10$  and  $< 50 \mu\text{m}$ . The location of ballast water uptake and therefore the species composition present will be a challenge for regulatory compliance testing.

Density of cells at L4 varies due to the environmental conditions throughout the year; having that also the relationship between number of cells and fluorescence will vary depending on the chlorophyll content and cells size in the community (Veldhuis et al., 1997; Bradie, 2016; Bradie et al., 2017). For the fluorometers, considering that the calibration factor is defined by the manufacturer, different equipment, regardless measuring the same fluorescence, may provide different results (Bradie et al, 2017). The use of filters will also incur in error as observed by Castro & Veldhuis (2018), where smaller cells ( $< 10 \mu\text{m}$ ) overestimated the number of cells in the 10 to  $50 \mu\text{m}$  size fraction by as much as a factor of 5.4.

#### **4.5. Conclusion**

Knowledge of phytoplankton viability allows the discrimination of functioning and non-functioning cells in the water column which is essential for regulatory ballast water issues. In the natural assemblage investigated (L4), the abundance of marine phytoplankton obeyed a seasonal pattern regardless of size ( $2 - 50 \mu\text{m}$ ). An overall good correlation was observed between the fluorometers measurements and in comparison with the flow cytometer. Flow cytometry analysis showed a potential over-estimation of the number of cells in particular when a high number of dead cells was found. Its use with DNA-specific dyes should be further investigated and precautions should be taken regarding the inclusion of contaminants and debris within the results.

For the shipboard trials, the techniques compared in this study (PAM fluorometer, ST fluorometer and staining microscopy) showed a large variation in the number of viable

cells and often between replicate samples. This variation implies that a sufficient high number of replicate samples need to be analysed. Nevertheless, the overall outcome indicated the same level of risk on all occasions for regulatory purposes.

## **Chapter 5 : Temporal changes in phytoplankton biomass and cellular properties; implications for the IMO Ballast Water Convention**

A version of this chapter has been published and is available online as:

**Castro, M. C. T., & Veldhuis, M. J.** (2018). Temporal changes in phytoplankton biomass and cellular properties; implications for the IMO Ballast Water Convention. *Environmental Technology*, 2018, Jan 24:1-12. DOI: <https://doi.org/10.1080/09593330.2017.1423117>.

## Abstract

At two locations, coastal waters of the Wadden Sea, the Netherlands and at the station L4 (Western Channel Observatory) in the English Channel, UK, the temporal size class distribution of the phytoplankton community was investigated with respect to the size classes identified by the International Maritime Organization's Ballast Water Management Convention. As part of this Convention, allowable discharge concentrations of organisms within classes were defined, with the lower size range (10-50  $\mu\text{m}$ ) consisting mainly of phytoplankton.

Traditional size fractionation methods that use nylon mesh filtration (10  $\mu\text{m}$  mesh) showed considerable size bias. On average 23.1% of the larger than 10  $\mu\text{m}$  cells were still present in the < 10  $\mu\text{m}$  filtrate but 21.8% of the smaller sized cells were also retained on the mesh. In particular the latter would result in an overestimate of the number of cells per mL by as much as a factor of 5.4.

Flow cytometry was applied to give the precise size classifications of each cell. Temporal measurements, covering an annual cycle, indicated that at both test sites the phytoplankton in the size range 2 to 50  $\mu\text{m}$  was dominated by the smaller sized phytoplankton (< 10  $\mu\text{m}$ ). In terms of number of cells that fit the  $\geq 10$  < 50  $\mu\text{m}$  size class these were on average only 3.6% and 2% in the Wadden Sea and the L4 sampling site, respectively. In terms of chlorophyll biomass they represent 28.7% and 12%, respectively. This was mainly caused by the cellular increase in chlorophyll concentration which increases in proportion to increasing cell size. In contrast, the mesh filtration method resulted in much higher chlorophyll values for the 10-50  $\mu\text{m}$  size range; 53.7% in the Wadden Sea and 38% at station L4. This overestimation appears

to be caused by cells in 6-10  $\mu\text{m}$  size range being retained on the mesh rather than passing through.

Present findings are relevant in the context of the size class distribution based on flow cytometry and semi-quantification using chlorophyll as proxy for cell density.

Keywords: Ballast Water Management Convention, Flow cytometry, Fluorometry, Chlorophyll, Phytoplankton

## **5.1. Introduction**

With respect to particle size distribution in nature there are several universal laws based on allometric distributions. In many cases they are based on size and physiological or metabolic properties (Litchman et al., 2007; Litchman et al., 2009). In the oceanic environment, a commonly accepted rule is that the numbers of organisms per unit of volume tend to increase exponentially with decreasing size (Van Valen, 1973; Isao et al, 1990; Irwin et al., 2006; White et al., 2007). Also, within a phyla or class, sizes can vary considerably. Phytoplankton varies by up to 6 orders of magnitude in size and up to 9 orders of magnitude in volume (Finkel et al., 2010). Associated with these differences maximum cell density (Agustí et al., 1990) and various cellular properties also co-vary similarly to chlorophyll (Geider et al., 1997) and even the size of the genome (Veldhuis et al., 1997).

This cell size to number relationship has recently received new interest as a result of the International Maritime Organization's Ballast Water Management Convention (BWMC) (IMO, 2004). In order to minimise the spread on non-indigenous organisms through ballast water, this Convention is limiting the number of living organisms in ships' ballast water discharges. To this end, the Convention has defined specific size range distributions including a size range of  $\geq 10$  to  $< 50$   $\mu\text{m}$ . In nature, this size range

tends to be dominated by phytoplankton in terms of numbers while other organisms (e.g. microzooplankton) are far less abundant (less than 5%, unpublished results). However, this only represents a small component of the whole size range compared to the entire range of phytoplankton sizes present in marine or fresh waters. The smallest known phytoplankton is only 0.7  $\mu\text{m}$  (*Prochlorococcus*) (Chisholm et al., 1988) but other species can reach up to >2 cm in the case of colonies or chains (Hoek et al., 1995). In the latter case and according to the Ballast Water Management Convention, the individual should be measured as it is the smallest unit able to reproduce [(IMO, 2008). The main reason for defining a regulation based on allowed concentrations of organisms in ships' ballast discharge has been the fact that many of the toxic or otherwise harmful phytoplankton species are found within this size category. However, a significant number of phytoplankton species, including bloom forming harmful algae, are smaller than 10  $\mu\text{m}$  (e.g. *Phaeocystis* spp., *Pfiesteria* spp. and *Chrysochromulina* spp.) (Gollasch et al, 2007). Small sized species also present higher growth rates, which may be an advantage when colonizing a new environment (Kagami & Urabe, 2001; Liebich et al., 2012).

Phytoplankton (or specific sub-populations of) biomass and dynamics are generally studied as a whole so the establishment of a fixed size range imposes new criteria on studies. With the exception of phytoplankton blooms, the defined size range has a relatively low numerical abundance relative to smaller sized cells (Chisholm, 1992). On the other hand, larger cells possess much higher concentrations of cellular chlorophyll, a cell component commonly used to estimate biomass or even cell density. As chlorophyll concentrations vary hugely with cell size, errors on cell density estimates based on chlorophyll concentrations will be significant. Even within a relatively small



size range of 10-50  $\mu\text{m}$  the diameter of the cell varies by a factor of 5 and, therefore, the volume of the cells (assuming they are spherical) will vary by a factor of 125.

The present study was conducted in order to examine the application of flow cytometry and fluorometry in characterizing natural phytoplankton communities with special attention to cell size. In addition, the annual variability of cellular properties like cell size and chlorophyll fluorescence combined with the actual size distribution of the cells was also investigated. The study covers a whole year at 2 different locations the Western Wadden Sea in the Netherlands and the Western English Channel in the UK.

## **5.2. Material and Methods**

### **5.2.1. Area of study**

#### ***5.2.1.1. Den Oever Harbour (the Netherlands)***

Water samples (ca. 1 L) at the test site in Den Oever (Western Wadden Sea, the Netherlands, 52°56.07'N; 05°02.19'E – Figure 5.1) were collected weekly during a full year (2016). The harbour is in the inner part of the Wadden Sea, a shallow estuary repeatedly influenced by fresh water input from a nearby lake (Lake IJssel). During the year the temperature varied from 1 to 22 °C, and nutrients ( $\text{PO}_4$ ,  $\text{NO}_3$  and silicate) were depleted from May until the end of September.

Whole samples and samples gently filtered over a 10  $\mu\text{m}$  mesh filter were analysed within 30 min of collection.

#### **5.2.1.2. L4 station (*Western English Channel, UK*)**

Samples were collected from the coastal station, L4, of the Western Channel Observatory (WCO) in the English Channel, about 13 km off Plymouth, in waters of approximately 50 metres deep (coordinates 50°15.0'N; 04°13.0'W – Figure 5.1) (Smyth et al., 2015). Relatively open sea characteristics may be found at the L4 site as well as features resulting from the influence of land with the inflow of water with higher concentrations of nutrients coming from rivers (Harvey, 1933; Pingree & Griffiths, 1978; Woodward et al., 2017).

L4 samples are collected on a weekly basis, weather permitting, for ongoing research projects conducted by the Plymouth Marine Laboratory (PML) and the Marine Biological Association (MBA). These are some of the longest time-series in the world for phytoplankton and zooplankton. In the present study L4 samples were collected from June 2016 to May 2017.

Water samples from L4 were collected from the surface using a bucket and were analysed immediately or, in a few exceptional instances, samples were kept in a constant temperature room (held at L4 seawater temperature) and were analysed within 18 hours after collection.

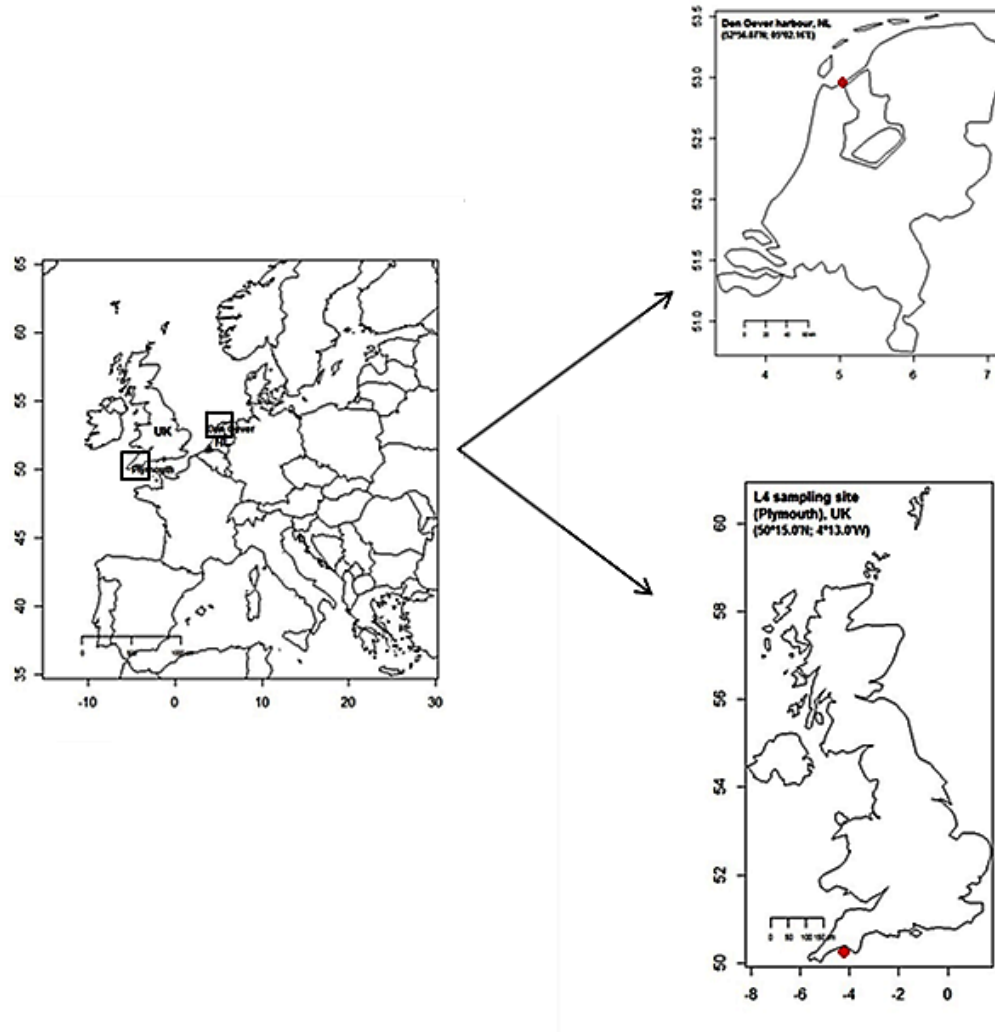


Figure 5.1. Study area showing Den Oever harbour in the Wadden Sea, The Netherlands ( $52^{\circ}56.07'N$ ;  $05^{\circ}02.19'E$ ) and the L4 sampling site in the Western English Channel, UK ( $50^{\circ}15.0'N$ ;  $04^{\circ}13.0'W$ ).

## 5.2.2. Methods

### 5.2.2.1. Flow cytometry

Flow cytometry (FCM) is widely applied in biological research including plant cells, yeast, phytoplankton bacteria and viruses (Davey & Kell, 1996, Legendre et al., 2001; Ormerod, 2009). In short, a set of bio-optical parameters is analysed from particles

passing a narrowly focussed laser beam. While passing the laser a variety of cell properties related to size (Forward Light Scatter: FS) optical density (Side Scatter: SS) of auto or induced fluorescence are generated by each individual cell. This is done as peak or integrated values, varying with instrumentation. This information can afterwards be analysed semi-quantitatively and allow selective visual clustering of cells with matching values.

Flow cytometric analyses were conducted using a Beckman Coulter (BC) EPICS-XL-MCL in Den Oever and with a Bektom Dickinson (BD) FACSort™ at PML. 2 mL samples were analysed in triplicate, with single values or averages of the triplicates being used for further analysis according to Veldhuis & Kraay (2000).

The settings of the instruments were adapted to display phytoplankton cells in the size range from 2 to 50  $\mu\text{m}$ . The size was measured as the scattered light in the forward direction (FS), the measurement best related to size (Ormerod, 2009). The red fluorescence from the phytoplankton chlorophyll was measured after excitation with blue laser light (488 nm) as autofluorescence of the chlorophyll pigment (emission > 630 nm).

Standard spherical beads with known diameters (9.7 and 50  $\mu\text{m}$ , Polysciences) were used as an internal standard for instrument calibration. These beads are uniform in size with known coefficients of variation (C.V. <2%) and measurements should possess the same spread for size and fluorescence.

Data analysis was based on clustering (sub) populations with identical size and chlorophyll fluorescence properties and considering the IMO size classifications, the fixed size defined implies that size rather than a specific population of cells was selected. Since phytoplankton populations usually have a broad size range, even within a species, the implication of this selection may be that only a part of the groups meets

the size requirement. Next to cluster analysis resulting in grouped average values of cell size and chlorophyll fluorescence a frequency distribution of the cell size of the entire phytoplankton population was also made. This was done by reducing the standard 1024 channels, covering 4 decades of variation in size, into a 256 channel logarithmic mode, i.e. increasing bin size at the larger size ranges.

#### **5.2.2.2. Fluorometry**

Samples collected in Den Oever were analysed for phytoplankton biomass, in terms of chlorophyll fluorescence and photosynthetic efficiency, after dark adaptation using a WALZ-Water-PAM fluorometer, equipped with a blue excitation LED according to Schreiber (Schreiber, 1998). The instrument was calibrated for background fluorescence using 0.2  $\mu\text{m}$  filtered water.

This analysis provides an estimate of the chlorophyll-a concentration of the total and  $<10\text{ }\mu\text{m}$  phytoplankton ( $F_0$  and  $F_{0<10}$ ). The difference between both values was used to calculate the chlorophyll-a fluorescence of the  $>10\text{ }\mu\text{m}$  ( $F_{0>10}$ ) fraction.

L4 samples were analysed using the Ballast Check 2 PAM fluorometer. This uses two measuring LEDs with multiple turnovers to determine organisms' photosynthetic activity. The equipment includes a filtration step (10  $\mu\text{m}$  mesh filter) and based on the measured variable fluorescence it provides an estimated abundance for cells  $>10\text{ }\mu\text{m}$  based on the conversion of a fluorescence value divided by a set constant value of fluorescence per cell. To estimate the total number of cells we used a 0.2  $\mu\text{m}$  filter. Therefore the calculated size fraction in this case is for cells smaller than 10  $\mu\text{m}$ .

A dark adaptation period of at least 15 minutes was always observed before analyses.

### **5.2.2.3. Size range determination**

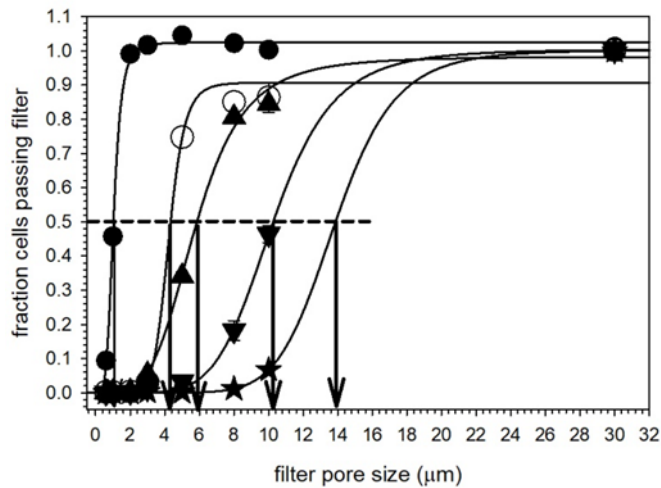
Internal standard beads (9.7 µm, Polysciences) were used to distinguish between two size classes of phytoplankton (sub) populations combined with a series of size fractionation experiments. These were conducted to establish the relationship between the arbitrary estimates of size, determined as the forward light scatter (FS), and size based on selective filtration.

A suspension of mono algal cultures and samples collected from the field, the latter with clearly distinguishable subpopulations, were gently filtered over a series of filters ranging from 20 to 0.2 µm. The 20 and 10 µm filters were nylon mesh filters with nucleopore filters (8, 5, 3, 2, 1 and 0.6 µm) being used for the subsequent filtration steps. During the sequential filtration steps great care was taken that some sample fluid remained on top of the filter and that the filter was not run dry thereby avoiding damage to cells. Three to five replicates of samples were analysed and the number of cells passing through each filter were counted using flow cytometry. Using a logistic (sigmoidal) fit the size, as estimated spherical diameter (ESD), of the cells was determined as the number relating to 50% retention on the filter according to equation below using SigmaPlot (version 12.5).

$$f = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

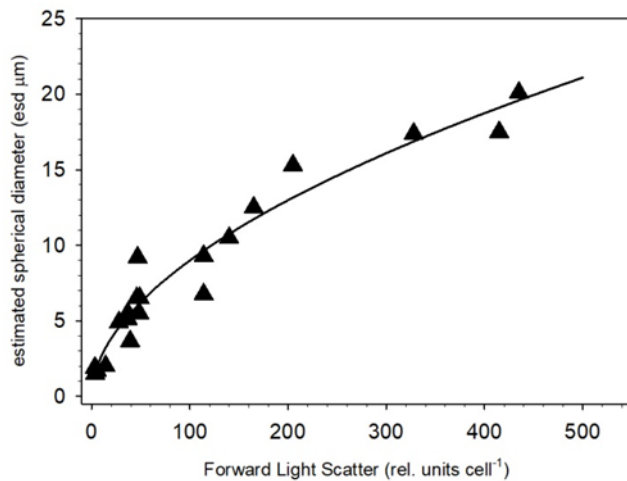
- Where  $x_0$  = infinitive pore size
- $x$  = pore size of filter applied
- $a$  and  $b$  computed constants
- $f$  = fraction of cells passing filter

Using this sigmoidal curve fit for each phytoplankton population the average size, as an ESD, was determined using a level of 50% of the population present (Figure 5.2). All cultures and field samples used had a length to width ratio of a factor of less than 3.



**Figure 5.2. Fraction of initial cell number of 4 different phytoplankton species remaining present in filtrate as a function of applied filter pore size. Lines are calculated fit of logistic function. Arrows are associated cell size based on 50% of cells present.**

In total 21 samples, sampled throughout the year in Den Oever, were fully analysed using flow cytometry and the estimated spherical diameter of the phytoplankton subpopulation was compared with the corresponding forward light scatter signal, as a proxy for cell size (Figure 5.3). No linear relationship was found but, based on the curve regression fit, the ESD of subpopulation or individual cells can be determined based on the FS measured.



**Figure 5.3. Forward light scatter versus size fractionated estimated spherical cell diameter.**

## **5.3. Results**

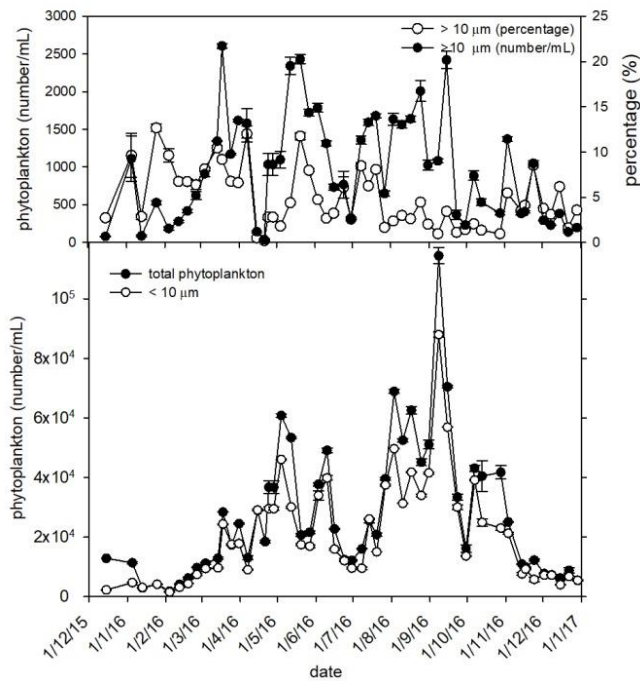
### **5.3.1. Temporal distribution**

Figure 5.4 (a) shows the annual distribution of phytoplankton numbers in Den Oever with a typical spring and autumn bloom and low cell density in the winter season. The phytoplankton community was throughout the year dominated by smaller sized (<10 μm) phytoplankton using the 9.7 μm reference beads as a selection criteria for size. In terms of cell density, the number of phytoplankton cells larger than 10 μm varied between 26 and 2662 cells per mL (annual average 982 cells/mL, table 5-1). Compared to the total number of phytoplankton cells measured, this size class was only a minor fraction of the total, ranging from 0.1 to 14 % (mean value of 3.6 %).

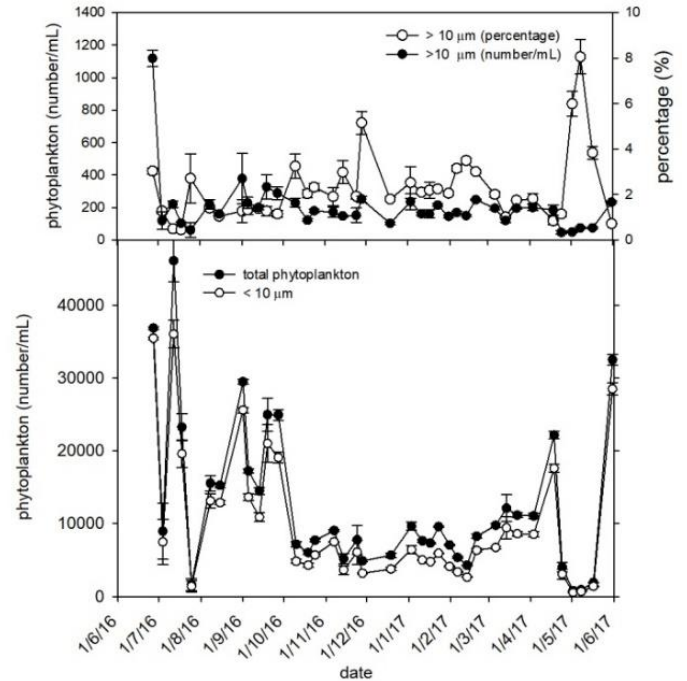
The L4 site (Figure 5.4 (b)) showed a similar trend with a distinct spring/summer and autumn blooms and lower numbers during the winter (October to March). Previous studies described the spring and autumn blooms composed mainly by diatoms whilst



dinoflagellates are dominant during the summer (Southward et al., 2004). The total number of cells per mL found for the whole period was 12590 in average, however cells larger than 10  $\mu\text{m}$  corresponded in average to only 201 cells/mL (CV%=  $\pm 87$ ) (table 5-1).



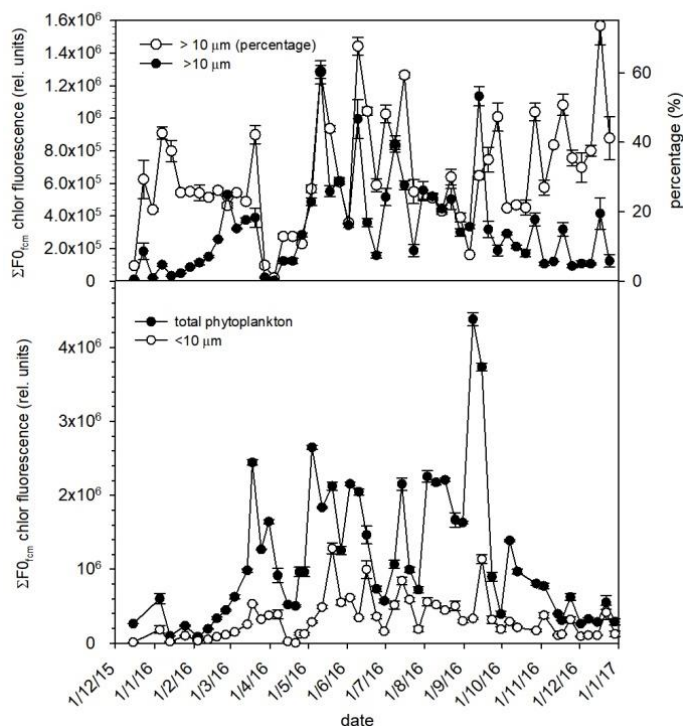
(a)



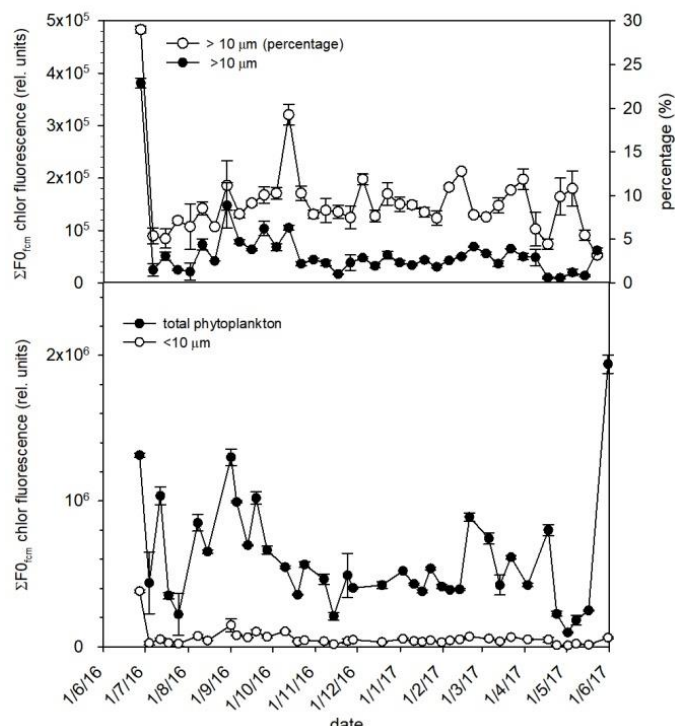
(b)

**Figure 5.4. Annual number of total phytoplankton and fraction <10  $\mu\text{m}$  (bottom graph). Number and percentage of phytoplankton cells in fraction >10  $\mu\text{m}$  (top graph): Den Oever (a) and L4 (b).**

Cell density, cell size of each individual cell and the chlorophyll auto-fluorescence ( $F_{0_{fcm}}$ ) of each cell were measured concurrently. The collective values of these cellular  $F_{0_{fcm}}$  values also provide an estimate of chlorophyll biomass (Figure 5.5 (a), Table 1). The percentage of chlorophyll associated with the larger cell sizes ( $> 10 \mu m$ ) varied considerably throughout the year in Den Oever, ranging from 0.8 to 80% of the total, but the annual mean value of 28.7% was higher than the value based on cell number. Chlorophyll biomass results measured using flow cytometry for L4 samples also showed a similar trend to the pattern found for number of cells (Figure 5.5 (b)). And, as experienced in Den Oever, results from the fraction larger than  $10 \mu m$  were higher because larger cells have higher chlorophyll content, showing an average of 12% ( $CV\% = \pm 108$ , Table 1), with values ranging from 3 to 30% of the total.



(a)



(b)

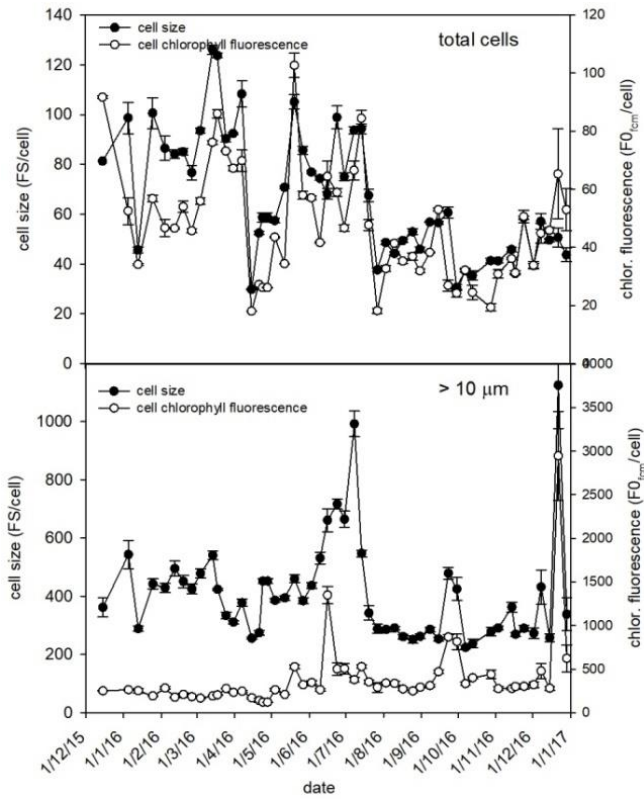
**Figure 5.5. Annual flow cytometric integrated chlorophyll concentration of total phytoplankton and fraction  $<10 \mu\text{m}$  (bottom graph). Integrated chlorophyll concentration and percentage of chlorophyll in fraction  $>10 \mu\text{m}$  (top graph): Den Oever (a) and L4 (b).**

The annual analysis of the cellular characteristics of the phytoplankton in Den Oever showed alongside to a variation in terms of numbers also changes in the cellular properties of size (FS) and chlorophyll autofluorescence ( $F0_{fcm}$ ) (Figure 5.6 (a)). For the total phytoplankton community these average values varied by as much as a factor of 4 for both size and chlorophyll throughout the year. Using the conversion of equation 1 the corresponding average sizes would range from 5 to 15  $\mu\text{m}$  (Figure 5.3).

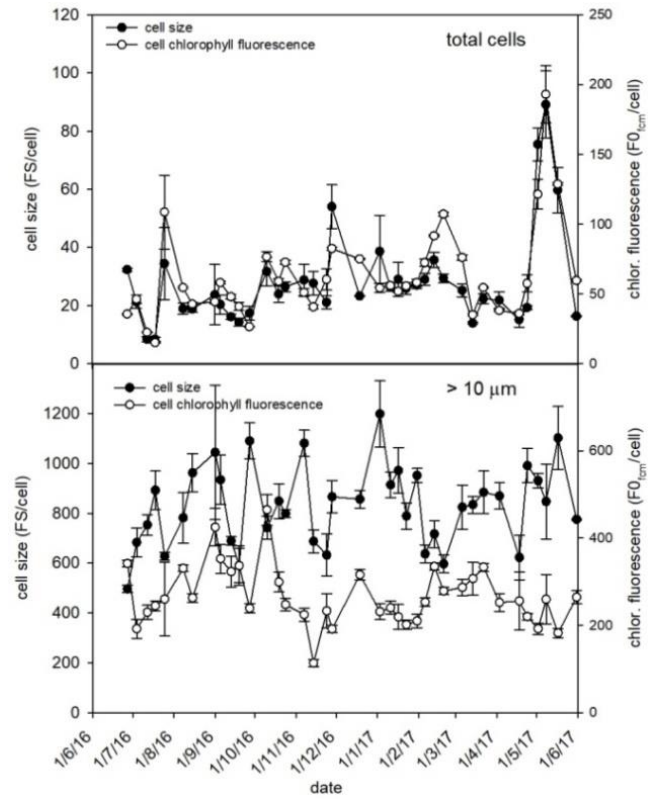
Identical results were measured at station L4 (Figure 5.6 (b)) where the minimum average value represented ca. one quarter of the average values found for cell size and

chlorophyll content. While the ratio between maximum and minimum single results varied by a factor of ca. 15 for both cellular properties.

On a more detailed level, and based on a clearly visible subpopulation, flow cytometrically derived values of size and chlorophyll also co-varied indicating a clear relationship between size and chlorophyll content (Figure 5.7). This relationship was found for all size classes covering the entire size range of phytoplankton cells in both sampling sites.

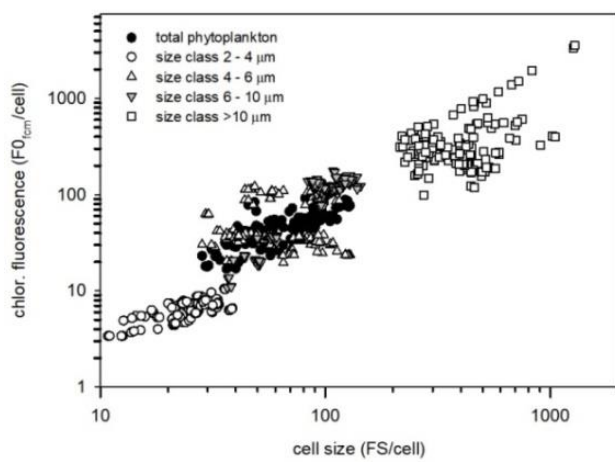


(a)

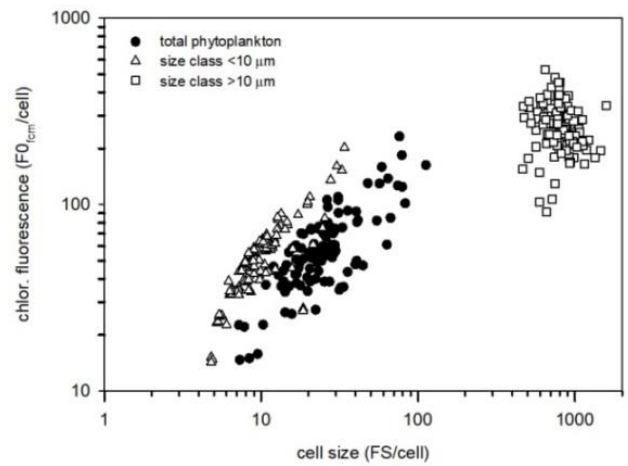


(b)

**Figure 5.6. annual variation in cell size and cellular chlorophyll autofluorescence of total phytoplankton community (2 - > 50  $\mu\text{m}$ ) and size class > 10  $\mu\text{m}$ : Den Oever (a) and L4 (b).**



(a)



(b)

**Figure 5.7. Covariation between cell size (measured as forward light scatter) and cellular chlorophyll autofluorescence of total phytoplankton community (2 - 50  $\mu\text{m}$ ) and different subpopulations 2 – 4  $\mu\text{m}$ , 4 to 7  $\mu\text{m}$ , 7 to 10  $\mu\text{m}$  and > 10  $\mu\text{m}$  (Den Oever data - a). Same covariation for the total number of cells (2 to 50  $\mu\text{m}$ ) and for organisms between 2 and 10  $\mu\text{m}$  and from 10 to 50  $\mu\text{m}$  (L4 data - b).**

**Table 5-1. Minimum/maximum and annual averages of flow cytometric measurements of total phytoplankton cells, integrated chlorophyll and relative contribution of fractions <10 and > 10 µm (based on flow cytometric separation of size, FS). Percentages are based on annual averages (top table: Den Oever; bottom table: L4).**

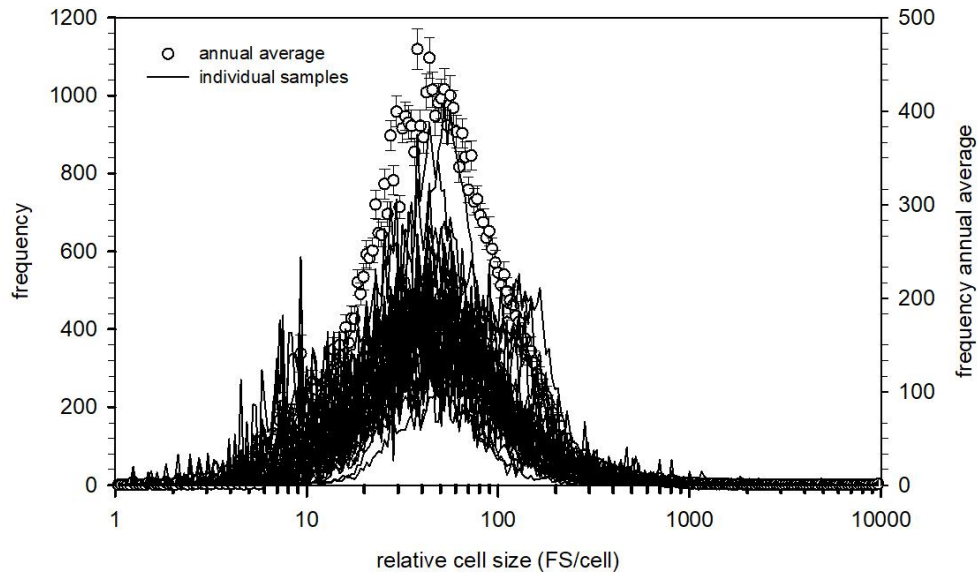
	phytoplankton [number/mL]				Integrated Chlor. [F0 <sub>FCM</sub> ]			
Size fraction	Min	Max	Average ± CV%	Number % of total	Min	Max	Integrated Chlor. ± CV%	Integrated Chlor. (%)
total	1788	119677	27042 ± 83		74400	4550000	1,146,072 ± 81	
FS <10 µm	1689	110345	26059 ± 75	96.3	48257	4203249	816,717 ± 94	71.3
FS >10 µm	26	2661	982 ± 73	3.6	63.0	274000	329,355 ± 85	28.7
	phytoplankton [number/mL]				Integrated Chlor. [F0 <sub>FCM</sub> ]			
Size fraction	Min	Max	Average ± CV%	Number % of total	Min	Max	Integrated Chlor. ± CV%	Integrated Chlor. CV%
total	307	49429	12590 ± 83.6		21523	2008952	595907 ± 63	
FS <10µm	213	38344	9988 ± 92.5	98	9526	1826536	422942 ± 77	88
FS >10µm	4	1173	201 ± 87.3	2	363	394890	57209 ± 108	12

### 5.3.2. Detailed size classification

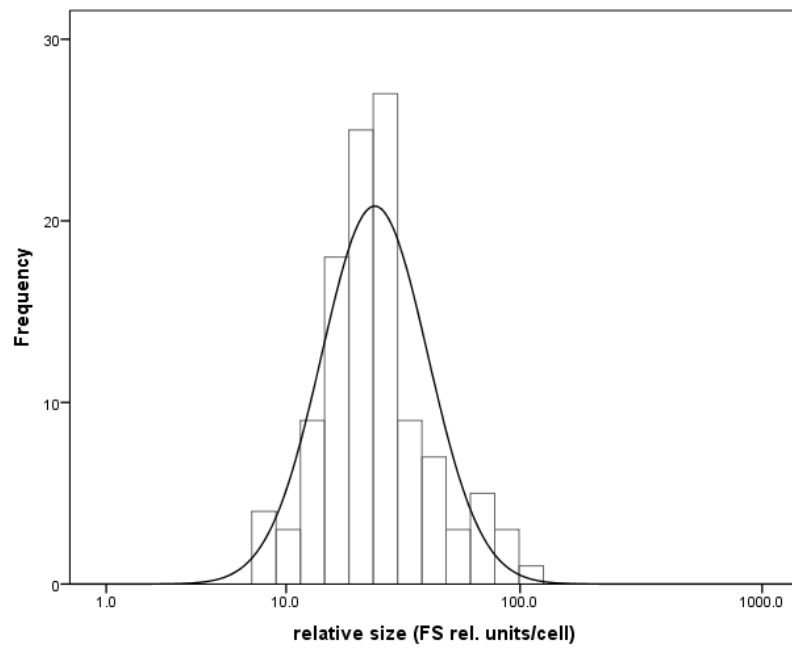
Because flow cytometry generates values of size and chlorophyll fluorescence data for each individual cell, it is possible to generate a complete frequency distribution of the size distribution of each sample analysed (Figure 5.8).

This was done based on a logarithmic distribution of the bin-size varying from 2.7 FS units at the lower size range to 340 FS units per bin of the largest bin. Throughout the year the size distribution of the phytoplankton community remained rather constant despite changes in absolute numbers. Only during typical bloom events, in spring or autumn, a relative increase in certain size ranges (6 to 8 and 10 to 15  $\mu\text{m}$ ) was observed. These were usually related to the episodic occurrence of blooms of mono-specific phytoplankton species. On the basis of the annually averaged values, the highest numbers of phytoplankton fall within the FS size range of 10 to 200  $\mu\text{m}$ , these values correspond with an ESD ranging from 2 to 20  $\mu\text{m}$  (Equation 5-1) (Figure 5.8 (a)). A frequency histogram of the observed FS values at station L4 is shown in Figure 5.8 (b). At this station the values of FS for total phytoplankton varied from 7.2 to 112.3 and were concentrated between 10 and 100 on a logarithmic distribution. Also from L4 data we can see a constant size distribution throughout the year regardless changes in absolute number.





(a)



(b)

**Figure 5.8.** Frequency distribution of cell size of each sampling day in Den Oever (average of 3 replicates, black lines) and annual average  $\pm 1$  sd (right scale) (a); and frequency distribution of cell size for total phytoplankton at station L4 considering all samples/replicates in the period ( $N=114$ ,  $FS= 28.2$   $CV\% = \pm 63$ ) (b).

### 5.3.3. Size fractionation 10 µm mesh

As mentioned, the IMO's ballast water performance standard (Regulation D-2) provided in the BWM Convention is defined on a size class basis. Having this in mind, the following section describes the results of the samples filtered over 10 µm mesh, the commonly applied method to separate size classes, compared to entire sample. Measurements of total phytoplankton biomass (PAM fluorescence,  $F_0$ ), cell density and FCM-integrated chlorophyll values based on the mesh separation method resulted in distinct differences when compared to those based on flow cytometric values for size (Figure 5.5; Table 5-2). On average, the values based on the filter screening were substantially higher for the >10 µm size fraction at both test sites.

Applying a standard fluorescent measurement showed that 53.7% of the total phytoplankton chlorophyll fluorescence (PAM- $F_0$ ) was associated with phytoplankton retained on the 10 µm mesh in Den Oever. Therefore the theoretical concentration of cells in the >10 µm size fraction would be 6148 cells/mL, or 22.7% of the total. This is 6.2 times higher than measured using flow cytometric size selection (982 cells/mL). The FCM-integrated chlorophyll measurements also showed that 47.3% of the chlorophyll was retained on the 10 µm mesh. This percentage is close to the value based on the bulk chlorophyll fluorescence (53.7%).

In addition to the whole water sample, a detailed flow cytometric analysis of size and cellular chlorophyll fluorescence was conducted on the fraction of phytoplankton passing the 10 µm mesh (Table 5-2). Analysis showed that both cells with a flow cytometric determined size of > 10 µm were passing the 10 µm mesh but also that smaller sized cells were retained on the filter.

On average 227 cells/mL were measured that were  $>10\ \mu\text{m}$  on basis of their size (FS) in the  $10\ \mu\text{m}$  mesh filtered water samples. Compared to the total number of phytoplankton cells this was only 1.1% but as much as 23.1% of the number in the same size range of cells in the unfiltered sample.

Alternatively, an average of 5392 cells/mL were retained on the nylon mesh corresponding to 20.6% of the total phytoplankton number classified on basis of the FS-size  $<10\ \mu\text{m}$ .

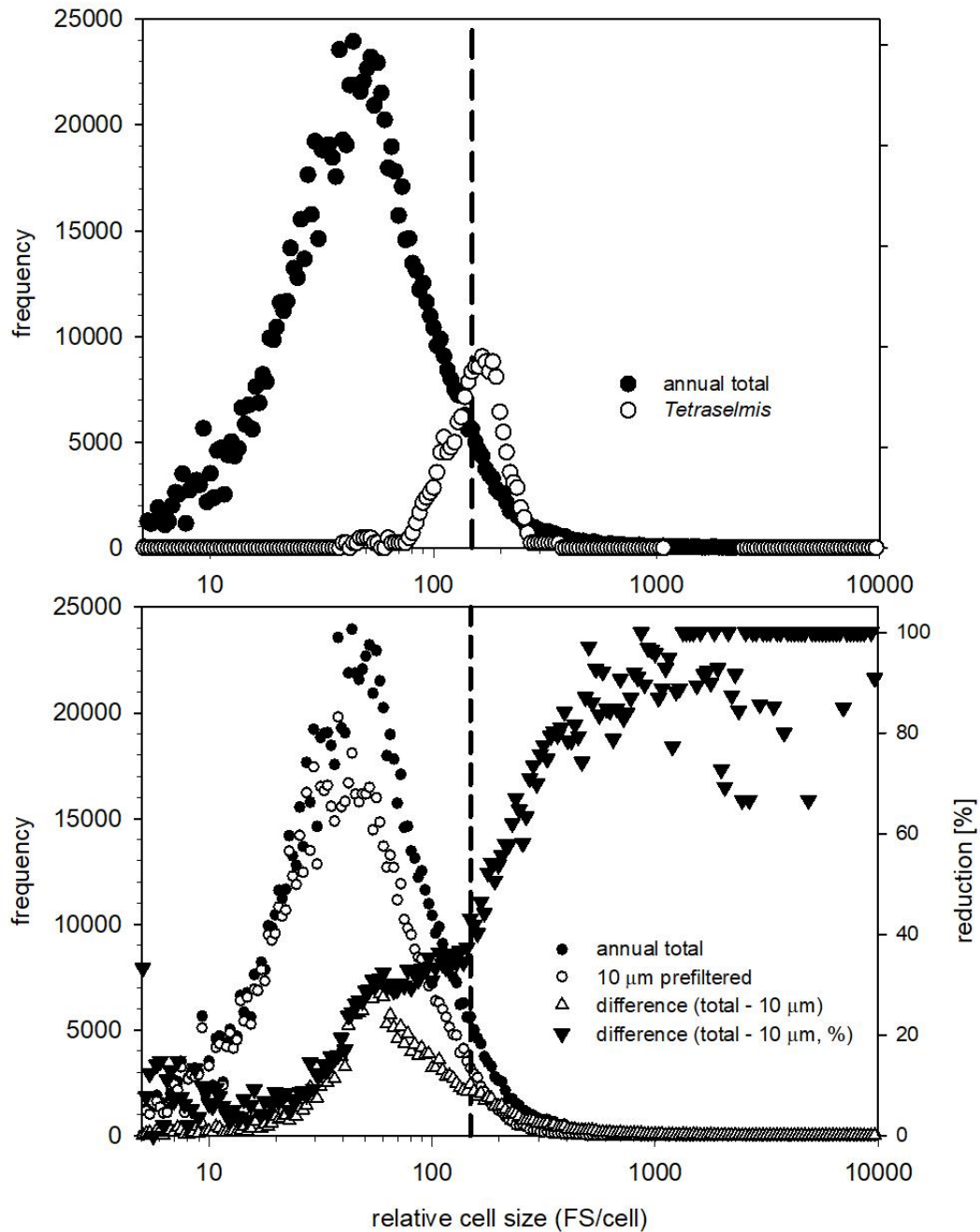
Applying the same procedure for the L4 data, resulting  $F_0$  measurements from the BC 2 fluorometer showed 38% of the total phytoplankton associated with the fraction over  $10\ \mu\text{m}$ , which would mean an average of 1812 cells/mL. This is 9 times higher than the 201 cells/mL average number detected with FCM and 14.4% of the total cells in average.

**Table 5-2. Minimum/maximum, annual averages including the coefficient of variation (CV) of unfiltered and 10 µm mesh filtered fraction of phytoplankton biomass (PAM-F0), total cell numbers, integrated chlorophyll values based on flow cytometric measurements, calculated number of cells and size classification on basis of FS values < or > 10 µm (top table: Den Oever; bottom table: L4).**

Den Oever											
		PAM-chlorophyll (F0)				phytoplankton [number/mL]			Integrated Chlor. [F0 <sub>FCM</sub> ]		
sample type	size fraction	F0 Chlor. biomass min	F0 Chlor. biomass max	average F0 Chlor. biomass ± CV%	F0 Chlor. % of unfiltered	phytoplankton number average ± CV%	phytoplankton number % of unfiltered	phytoplankton number > 10 µm (%)	Integrated Chlor. ± CV%	Integrated chlor % of unfiltered	> 10 µm Integrated chlor (%)
unfiltered	total	81	17503	503 ± 66		27,042 ± 83			1,146,072 ± 81		
	FS <10 µm					26,059 ± 75	96		816,717 ± 94	71.3	
	FS >10 µm					982 ± 73	4	3.6	329,355 ± 85	28.7	28.7
10 µm mesh filtered	total (<10 µm)	32	888	232 ± 76	46	20,894 ± 82	77		603,719 ± 94	52.7	
>10 µm mesh filtered	calculated			270 ± 73	54	6148 ± 81	23	22.7	542,353 ± 79	47.3	47.3
	FS <10 µm					20,667 ± 89	76		554,539 ± 86	48.4	
	FS >10 µm					227 ± 86	0.8	1.1 (23)	49,180 ± 101	4.3	8.1 (14.9)
L4											
Sample type	Size fraction	F0 Chlor. Biomass min	F0 Chlor. Biomass max	Average F0 Chlor. Biomass ± CV%	F0 Chlor. % of unfiltered	Phytoplankton number average ± CV%	Phytoplankton number % of unfiltered	Integrated Chl of unfiltered ± CV%			
Unfiltered	total	122	3767	793 ± 70.3		12590 ± 83.6		595907 ± 63			
	FS <10 µm					9988 ± 92.5	98	422942 ± 77			
	FS >10 µm					201 ± 87.3	2	57209 ± 108			
< 10µm mesh filtered	Calculated	20	3582	575 ± 87.4	61.8	10777	85.6	369462			
10µm mesh filtered	(>10µm)	2	992	355 ± 83.5	38	1812	14.4	226445			

The effect of the 10  $\mu\text{m}$  mesh filtration on the size distribution of the phytoplankton was also analysed on the level of each individual cell for samples collected in Den Oever, similar to that shown in Figure 5.8 (a) for the unfiltered sample. For this analysis, the frequency distribution of the cell density for the entire year was integrated instead of using the annual average (Figure 5.9). The top graph of this figure shows the size distribution of a cell culture of *Tetraselmis* sp. (average cell diameter of 12  $\mu\text{m}$ ). The detailed cell size analysis showed that, as commonly observed for phytoplankton, the population of *Tetraselmis* was far from uniform in size distribution and varied by as much as a factor 3. The size of 95% of the cells varied between values for FS of 80 and 240.

The cell size distribution of the 10  $\mu\text{m}$  mesh filtered sample showed values matching those of the unfiltered sample in the lower range of cell sizes up to an FS value of 40. With increasing cell size, the discrepancy in numbers between total and mesh filtered increased even at values of FS below the value corresponding with a cell size of 10  $\mu\text{m}$ . Above an FS value of 50 the numerical difference between the total and mesh filtered water declined again with increasing cell size. In terms of percentage of reduction in cell density due to the filtration a different trend was observed. At the lowest size ranges the difference was in the order of a few percent increasing to as much as 35% at the value of FS corresponding to a size of 10  $\mu\text{m}$ . At the higher cell sizes this percentage increased rapidly. A near 100% reduction was only measured when the value of FS was higher than a value of 500.



**Figure 5.9.** Flow cytometric data of frequency distribution of phytoplankton cell size (2 - > 50 µm) of total phytoplankton (top graph), cells passing 10 µm mesh, numerical difference and percentage of difference between both data sets. Values are based on annually integrated numbers (below). Top graph is total phytoplankton and *Tetraselmis* sp. as a reference phytoplankton species. Dashed line indicates FS value corresponding with ESD of 10 µm.

## 5.4. Discussion

This study shows that flow cytometry is a useful, fast, accurate and reproducible tool for the size analysis of phytoplankton cells. Size distribution can be done based on the whole community, subpopulation and even as in this case down to the level of the individual cell. Although the data for size are usually based on arbitrary units of forward light scatter (Veldhuis et al., 1997; Ormerod, 2009) they can be converted into more realistic values of cell size using simple conversion factors.

Jennings & Parslow (1988) defined equivalent spherical diameter as the diameter of a sphere that would perform in the same way as the non-spherical particles presented in the sample; the authors highlight that the resultant dimension is always less than the true major dimension though. These conversions not only rely on the shape/dimensions and their conversion into a forward light scattering signal but also on instrumental differences in how the particle's cross-sectional area is determined (Karl-Boss et al., 2007) therefore indicating the need of proper calibration. The measurements also indicate that even within a single phytoplankton species (e.g. *Tetraselmis*) the variation in size can be considerable, as microscopic analysis confirmed. For many species of phytoplankton analysed the coefficient of variation of size ranged typically between 40 and 60%. The variability in the dimension of size is therefore natural but also explains the overlap when multiple species are present as in the current samples.

Infrequently and during blooms of selective species higher numbers of certain size classes are seen e.g. *Phaeocystis* or diatoms in the spring in the Wadden Sea (Cadée & Hegeman, 1991; Philippart et al., 2011). On an annual time scale these blooms are of minor effect on the general pattern of size versus cell number distributions (Figure 5.8). At station L4, Tarran & Bruun (2015) described periods of higher abundance for pico- and nanoplankton during the spring / summer in the top 20 meters. The summer peak is

probably encouraged by the summer thermocline breakdown allowing the mix of nutrients in the surface layers (Smyth et al., 2010; Tarran & Bruun, 2015). Samples from 07 April, 2017 showed the first signs of spring bloom arriving earlier than in recent years, confirmed in subsequent sampling to be dominated by *Guinardia delicatula* (Dr. Claire Widdicombe - personal communication). L4 results in early May showed very low numbers of cells per mL, probably as a result of being deprived of the nutrients that were consumed by the phytoplankton during the early spring bloom (L4 buoy data – PML Western Channel Observatory Blog - <http://www.westernchannelobservatory.org.uk/blog/?p=870>). (Figures 5.4 and 5.5).

These data also confirm the general trend that phytoplankton populations show an inverse relationship between numerical abundance and cell size as has previously been reported in the literature (Cermeño & Figueiras, 2009; Huete-Ortega et al., 2010; Álvarez et al., 2011).

On an annual basis, smaller sized cells were dominant at all periods not only at the more off-shore station (of the two in this study) L4 (13 Km off Plymouth with influence of the North Atlantic Ocean) but also nearshore in an estuary (Table 5.1). This dominance is not only restricted to certain periods like the summer when nutrients are normally low (Woodward et al., 2017) but throughout the whole year. Applying the size classification based on the conversion of flow cytometric derived values of size into ESD shows that on average, the relative contribution of cells with a dimension of 10 µm or larger to the total cell number was low, only 3.6% or 2% for the Wadden Sea and station L4, respectively. Even when taking into account the higher chlorophyll *a* concentration of larger sized cells only 28.7% (Wadden Sea) or 12% (L4) of the chlorophyll is associated with the larger size fraction.

Figure 5.8 shows that throughout the year and irrespective of the location, coastal or more open ocean, the flow cytometric determination of cell size results in a uniform and



continuing pattern of size distribution of the phytoplankton covering the entire range from 2 to > 50  $\mu\text{m}$  in diameter. As a result, classification of populations in terms of size classes will therefore be a rather arbitrary exercise. In this study up to 44% of the *Tetraselmis* population must be classified as cells with a diameter of less than 10  $\mu\text{m}$  (Figure 5.9). The rather strict definition used by the Ballast Water Management Convention (IMO, 2004) of 'minimum cell dimension' would imply that even within a single species, individual cells would not meet the criteria and exact sizing of all cells would be required. While flow cytometry provides a full-scale analysis of cell size in a time span of several minutes, more detailed microscopic analysis of a large number of cells would take many hours.

Our data also show that the commonly applied method of size selection by means of mesh filtration resulted in significantly different results. Reanalysis of the filtered fraction indicated that as much as 23.1% of the cells or 14.9% of the chlorophyll ( $F0_{\text{FCM}}$ ) of phytoplankton cells larger than 10  $\mu\text{m}$  passed through the mesh filter. In contrast, 21.8% of the cells and 43.0% of the chlorophyll of phytoplankton cells with estimated cell size <10 $\mu\text{m}$  were retained on the mesh filter. In particular, the bias towards smaller sized cells by the filter resulted in an overestimation of the actual numbers by as much as a factor of 5.4. Also in terms of chlorophyll biomass the difference between both size selection methods was considerable. The direct measurement of chlorophyll biomass, applying PAM-fluorescence analysis, resulted in 53.7% and 38% of the chlorophyll associated with the larger sized cells in Den Oever and L4 respectively. These values were comparable with the flow cytometric data of differences in chlorophyll (47.3% and 29% in Den Oever and L4 respectively). In reality the actual number of cells was much lower, on average only 982 per mL (Den Oever) and 201 per mL (L4); and therefore also their chlorophyll concentration ( $F0_{\text{FCM}}$  of 28.7 and 12% in Den Oever and L4 respectively).

Finally, we return to the initial questioning on what would be the effect of cell size on the conversion factors used by fluorometers to convert fluorescence into cell density. In theory, any fluorescence parameter may be converted into number of cells/mL by means of an internal coefficient. However, variations in the size of cells and therefore in the content of chlorophyll within the cell can affect the fluorescence signal measured, which means that a simple conversion value based on chlorophyll fluorescence might not be completely reliable (Veldhuis et al., 1997; Bradie, 2016; Bradie et al., 2017). Another aspect to be considered is the device behaviour to a large number of smaller cells ( $<10\text{ }\mu\text{m}$ ), would their fluorescence signal influence fluorometer's numeric results? Since many fluorometers used in the quantification of the IMO relevant size class of  $10 - 50\text{ }\mu\text{m}$  include a filtration step, the bias due to smaller size cells retained on the filter may be significant.

On the other hand, based on an uniform distribution of size and numbers, an average cell size and accompanying chlorophyll fluorescence can be calculated. For the Den Oever test site the average cell size, as ESD, determined was  $20.8\text{ }\mu\text{m}$  ( $\text{CV} \pm 44\%$ , ranging from  $11.6$  to  $30.0\text{ }\mu\text{m}$ ) and corresponding chlorophyll fluorescence of  $329.4\text{ }F_{0\text{FCM}}/\text{cell}$  ( $\text{CV} \pm 85\%$ , ranging from  $49.4$  to  $608.7\text{ }F_{0\text{FCM}}/\text{cell}$ ). But even for the given coefficient of variation there would be a 12-fold variation in cellular chlorophyll, and with a fixed conversion factor an equal variation in corresponding number of cells.

## **5.5. Conclusion**

This study attempts to add value to the discussion on the possible implications resulting from cellular properties and biomass changes on the IMO's Ballast Water Convention implementation notably on the ballast water performance standard (Regulation D-2). Additionally, it intends to raise the issue of potential sources of error for further refinement of the instruments regarding a relatively unknown area where portable tools developed for

verifying ships' compliance to the BWMC may produce dubious / false results (Byllaardt et al., 2017).

In an ocean threatened by increasing CO<sub>2</sub> and many other natural and anthropogenic stressors, cell size composition will be affected and therefore the phytoplankton community structure (Finkel et al., 2010). This will pose additional challenges for indicative tools developed to measure abundance of photoautotrophic cells in the water. Accordingly, conversion factors based on photosynthetic activity will need to be robust enough to face the challenges of a changing ocean.

Sampling and analysis of ballast water samples is supposed to be a relatively rare procedure according to the tiered regulatory enforcement approach agreed at IMO. However, considering the challenges, there remains a feeling that there is not enough discussion and research to provide the needed confidence that is required of ballast water monitoring techniques.

## **Chapter 6 : Hyposalinity as a biosecurity tool for minimizing biofouling in ships sea-chests**

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## Abstract

Biofouling is a major vector in the transfer of non-native species around the world. Species can be transported on virtually all of the submerged areas on ships (e.g. hulls, sea-chests, propellers) and so antifouling systems are used to reduce fouling. However, with increased regulation of biocides used in antifoul coatings (e.g. International Maritime Organization tributyltin ban in 2008), there is a need to find efficient and sustainable alternatives. Here, we tested the hypothesis that short doses of low salinity water could be used to kill fouling species in sea-chests. Settlement panels were suspended at 1.5 m depth in a Plymouth marina for 24 months by which time they had developed mature naturally occurring biofouling assemblages. We exposed these panels to three different salinities (7 psu, 20 psu and Control (33 psu)) for two hours based on an in situ pilot study using a model sea-chest that we placed in the marina and flushed with freshwater. The salinity decreased from around 33 to 7 psu after ca. 80 minutes of freshwater inflow and then stayed at 7 psu in our model sea-chest trial. Fouling organism diversity and abundance was assessed before panels were treated, then immediately after treatment, and again one week and one month after treatment. Many of the native ascidian *Dendrodoa grossularia* appeared to survive but all other macrobenthos was killed by the 7 psu treatments after one week. The 20 psu treatments were not effective at killing the majority of fouling organisms. We propose that sea-chests be flushed with freshwater for at least two hours before ships leave port. This would not cause unnecessary delays or costs to ships, and would be a major step in improving biosecurity.

Keywords: marine fouling, shipping, non-native species, osmotic regulation, saline treatment

## 6.1. Introduction

Biofouling is considered a highly efficient vector in the transference of non-native species around the world (Carlton et al., 1995; Ruiz et al., 1997; Gollasch, 2002; Coutts & Taylor, 2004; Castro et al., 2016). Species can be transported on virtually all submerged areas of ships (e.g. hulls, sea-chests, propellers) and one common and efficient way of preventing and minimizing the fouling is the use of anti-fouling systems. However, some areas on ships hulls such as sea-chests and chain lockers are difficult to access and coat with anti-foulants. Consequently, these areas often get heavily fouled by a wide variety of marine organisms such as hydroids, serpulid polychaetes, barnacles, mussels, bryozoans and tunicates (Coutts & Taylor, 2004; Murray et al., 2011). Given the increasing importance of biofouling as a vector for non-native species introduction and spread due to the opening of new trade routes, climate change and the increasingly speed of vessels, among others facts, the International Maritime Organization decided to tackle the problems initially by adopting a set of voluntary regulations. In 2011 the International Maritime Organization Marine Environment Protection Committee issued the Resolution MEPC.207(62) outlining measures to minimise the risk associated with ship biofouling. These regulations are directed at many stakeholders (e.g. States, shipmasters, operators and owners, shipbuilders, port authorities, ship repair, dry-docking and recycling facilities, classification societies, anti-fouling paint manufacturers / suppliers) and other interested parties. Two subsequent sets of guidance on biofouling were released in subsequent years: one for recreational crafts less than 24 meters in length (MEPC.1/Circ.792, 2012), adopted in 2012, and the second evaluating the 2011 Guidelines for the control and management of ships biofouling to minimize the transfer of invasive aquatic species (MEPC.1/Circ.811, 2013) (Castro, 2014).

Following the entry into force of the Ballast Water Convention on 8 of September, 2017, it seems probable that ship's biofouling may become the subject of a new international treaty in a not so distant future. In May, 2017, a programme called "Building Partnerships to Assist Developing Countries to Minimize the Impacts from Aquatic Biofouling" (GloFouling Partnerships) was approved by the Global Environment Facility (GEF) to be implemented by the United Nations Development Programme (UNDP) and executed by the IMO. Its implementation phase is planned to start in the second half of 2018 with a duration period of five years (IMO Circular Letter No 3768). In some countries, biofouling management plans and record books are already in place as part of national regulations (e.g. in the United States of America, Australia and New Zealand). For instance, in the State of California (USA), ship owner/operators of vessels of 300 gross tons or larger need to answer eleven questions about hull husbandry every year (Scianni et al., 2013). Biofouling increases shipping operational costs; even microbial fouling, which is a pre-cursor to macro-fouling, increases fuel consumption due to frictional drag. There are also the costs of hull cleaning and painting (Schultz et al., 2011; Dobretsov et al., 2013; Davidson et al., 2016). Some organisms (e.g. bryozoans and algae) are tolerant to antifouling compounds and can grow on freshly applied antifouling paint, and are subsequently used as a substratum for other species (Murray et al., 2011). With the ban of tributyltin in 2008, other anti-fouling systems started to be used, being copper-based ones the most commonly used nowadays. Apart from copper, booster biocides are also used in antifouling system despite their potential impacts on the marine ecosystems (Faÿ et al, 2010; Price and Readman, 2013), for instance, glycerophospholipids from soybeans are considered effective booster biocides in antifouling paint (Batista et al, 2015). Antifouling compounds from marine bacteria, cyanobacteria, fungi as well as eukaryotic organisms have also been developed as biocides (Dobretsov et al., 2013). In terms of mechanical tools to remove biofouling, Hearin et al. (2016) showed that mechanical grooming is helpful in reducing

fouling on submerged surfaces coated with fouling-release coatings. Niche areas on vessels hulls (e.g. gratings and propellers) represent a great challenge to minimising biofouling on ships. On larger vessels, ships sea-chests were conceived to maximize seawater inflow (e.g. for internal cooling systems and ballast water). These box-shaped structures are difficult to access and coat, have lots of edges and welds, becoming sheltered areas for organisms to settle and recruit, therefore representing higher risk areas for biofouling accumulation (Coutts & Dodgshun 2007). In Canada, a study of 82 sea-chests from commercial ships showed that 80% of them had some level of fouling organisms and almost half included non-native species among the fouling assemblages (Frey et al., 2014).

Setting biosecurity goals and implementing measures for controlling non-indigenous species helps to avoid their spread (Collin et al., 2015). Numerous methods are available, for example ultraviolet light (Titus & Ryskiewich, 1994), heated water and steam (Leach, 2011; Piola & Hopkins, 2012; Growcott et al., 2016) or soaking areas in acids (e.g. acetic acid) or alkalines, such as hydrated lime (Rolheiser et al. (2012). In Alaska, the invasive colonial ascidian *Didemnum vexillum* was exposed to various treatments using acetic acid, bleach, freshwater or brine with 100% mortality when exposed to freshwater for four hours (McCann et al., 2013). In Brazil, Moreira et al. (2014) tested the use of freshwater to combat the spread of invasive corals *Tubastraea tagusensis* and *T. coccinea*. For both these species, two hour exposure to fresh water killed all the corals and this treatment is now routinely used for combat the spread of *Tubastraea spp.* on oil industry infrastructure. In New Zealand, Jute & Dunphy (2016) showed that two hour exposure to fresh water killed the invasive Mediterranean fan worm *Sabella spallanzanii*, while in hypersaline conditions (50 psu) 100% mortality was reached after 24 hours. Finally two studies conducted in Plymouth, UK, showed that low saline treatments can be highly effective at reducing biofouling and can be used in conjunction with anti-fouling coating systems

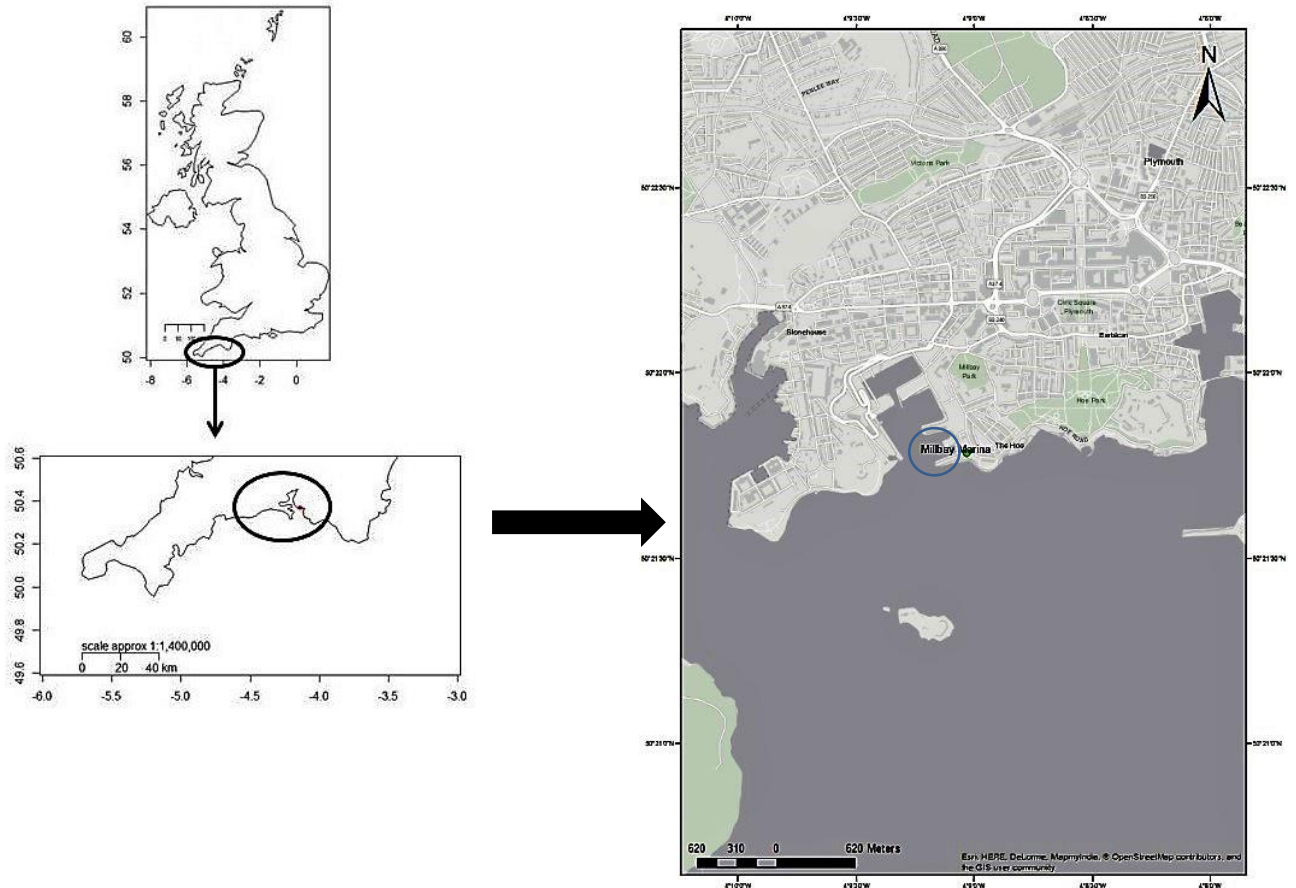


(Minto, 2014; Quinton, 2014). Although chemical treatments, the use of heat, or the use of UV light all work they can be costly, or pose health and safety risks and also increase corrosion of hulls. On the other hand, freshwater is not dangerous, and it is cheap and widely available. Given the importance of biofouling as a vector in the world transfer and spread of non-native species, this study tested the hypothesis that a rapid change in the salinity can kill fouling species taking into account the regulation of the osmotic pressure between the surrounded aquatic environment and the organisms body fluids and offers a simple and efficient biosecurity management tool to minimise biofouling in ship sea-chests. This case of study was conducted in southwestern England and is representative of the fouling community of the northeastern Atlantic Ocean.

## **6.2. Methods**

### **6.2.1. Study area**

The experiment was conducted in two phases, the first phase occurred in November/2016 and the second experimental phase during July/August 2017 at Millbay Marina (50°21'47"N; 004°09'02"W), in Plymouth, UK (Figure 6.1). The chosen marina is a semi-enclosed tidal marina open to the Plymouth Sound, a large bay on the south coast of Devon (Southwest of England). This bay is connected by a southern opening to the English Channel and is sheltered by means of an artificial breakwater that minimises the effects of prevailing southwestern swells and local wind waves predominant in the area (Bremekamp, 2012).



**Figure 6.1. Area of study (Millbay Marina, Plymouth, UK).**

### **6.2.2. Research design**

A model sea-chest was built to find out the lowest steady salinity that could be achieved when the chest was flushed with freshwater whilst submerged and open to surrounding seawater. The sea-chest was a polypropylene container of 80 Litres (external dimensions: 600 x 400 x 420 mm); 12 panels were fixed inside with stainless threaded rods to simulate gratings (Figure 6.2). A YSI 556 Multiparameter meter, complete with conductivity probe, was hooked inside the box to measure salinity. The box was deployed so that the panels

were vertical and about 1.5 metre from the seawater surface; measurements of temperature and salinity started immediately after the deployment and were recorded every 10 seconds. To create a hyposaline environment inside our immersed sea-chest, freshwater was flushed into the box through a hose connected to a tap on the pontoon. A flow rate of approximately 8 litres / min was kept during the experiment bearing in mind the necessity of preventing excess turbulence inside the box. Flow was suspended after 86 minutes when the salinity stabilised and the probe stopped recording five hours later.

Polyvinyl chloride (PVC) settlement panels (each 12 x 12 x 0.5 cm) were deployed in the same marina more than two years before this experiment, in June, 2014. Initially they were fixed in grids horizontally orientated with the roughened side facing outwards, in a depth of approximately 1.5 m, avoiding sedimentation and algae growth (Quinton, 2014). Five months before the low salinity experiment, panels were rearranged in a vertical position tied to a rope and attached to the pontoon. At this stage, panels were less exposed to the light, almost under the pontoon which also helped to preclude macroalgae. Fifteen of these panels were selected based on the existence of a well-developed fouling community, including the native ascidian *Dendrodoa grossularia* on all panels and the non-native encrusting bryozoan *Watersipora subatra* on most of the panels. The objective was to examine the effects of low salinity water treatments on the whole community assemblage on each panel.

Panels were subjected to one of the following treatments: 7 psu, 20 psu and control (33 psu) for two hours with five panels per treatment. The lowest salinity (7 psu) was chosen as it was the lowest steady value achieved inside our simulated sea-chest. The exposure time was chosen based on the studies conducted by Moreira et al. (2014) and Jute & Dunphy (2016). On the day before the experiment started, water from the marina was collected and stored in a constant temperature room of around 16°C at Plymouth Marine Laboratory similar to the temperature found in the marina. The water used to prepare the

different salinity treatments during the experiment was a mix of local sea water and pure fresh water (Milli-Q water), stored in the same room.

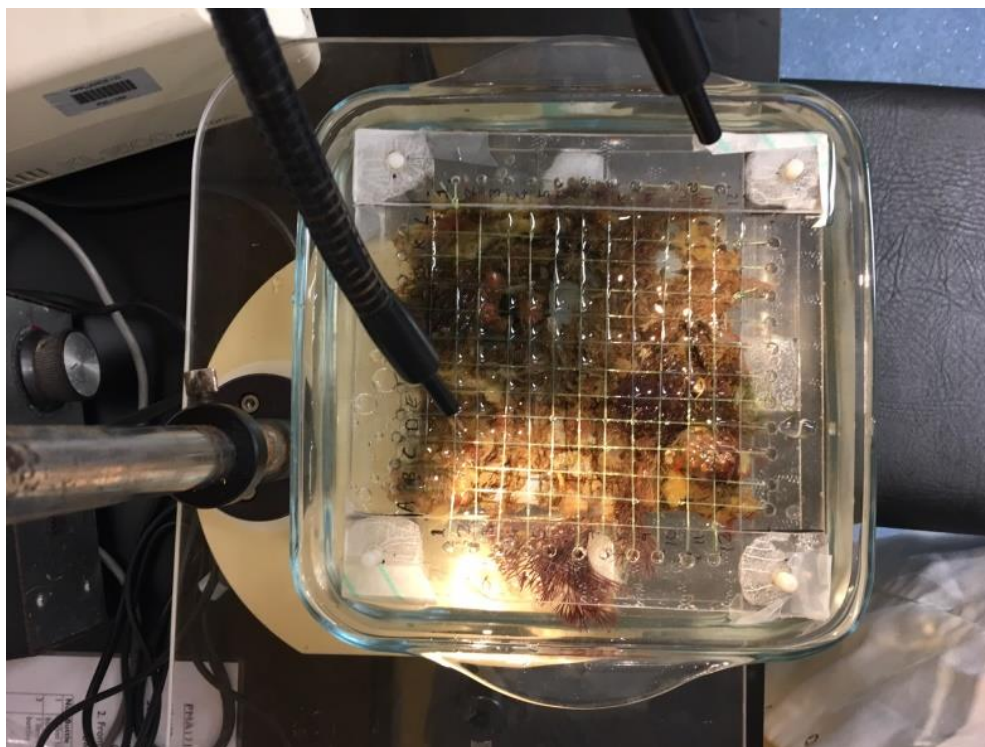


**Figure 6.2.** The simulated sea-chest built for the experiment (external and internal views).

### **6.2.3. Analysis**

An acrylic 12 x 12 cm quadrat divided into a 1 cm<sup>2</sup> square grid was used to enumerate organisms on the settlement panels. The apparatus (settlement panel & quadrat) were submerged in seawater in a Pyrex dish for analysis (Figure 6.3). At each intersection point on the grid, organisms were identified, where possible to species level. Each taxon was enumerated, with colonial invertebrates counted as one maximum per square. Analysis times were set to a maximum of 25 minutes in order to minimise stress to the organisms. Panels were evaluated regarding the abundance and mortality of fouling organisms before the exposure to fresh water, immediately afterwards, and on two more occasions: one week and one month after. Mortality was assessed e.g. through detachment of the organisms from the panels, a lack of response (e.g. tunicates with no reaction when siphons were touched), absence of zooids in erect bryozoans, alterations in the texture / colour of the organisms.

Data from fouling communities were entered into PRIMER-E for abundance analysis and were square root transformed prior to clustering analysis according to Clarke et al., 2016. Dendrogram plots were used to determine similarity of fouling communities before, immediately after, one week and one month after the exposure to one of three salinities targeted by this experiment.

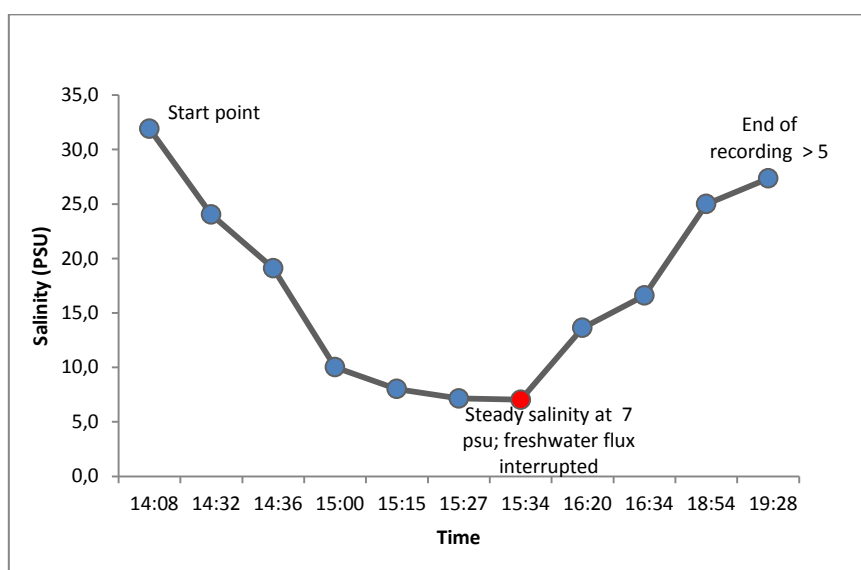


**Figure 6.3.** The apparatus (settlement panel & quadrat) submerged in seawater in a Pyrex dish ready for analysis.

### **6.3. Results**

The first phase of the experiment was to ascertain the lowest salinity value achievable inside our simulated sea-chest. The inflow of freshwater from the pontoon took 86 minutes to reach a steady salinity inside the box. Initial salinity was measured ca 32 psu at 14:08 hours; after 25 minutes the salinity decreased to 24 psu. One hour after the start of the experiment, the salinity had decreased to around 9 psu. At 15:27 hours the salinity

stabilized at 7 psu. The source of FW was then suspended at 15:34 hours and within one hour the salinity inside the box increased to around 16 psu; at 18:54 hours it was already 25. Five hours and 20 minutes later, the salinity was 27.3 psu, when the record was finished. During the experiment, water temperature varied between 13 and 13.6°C (Figure 6.4).

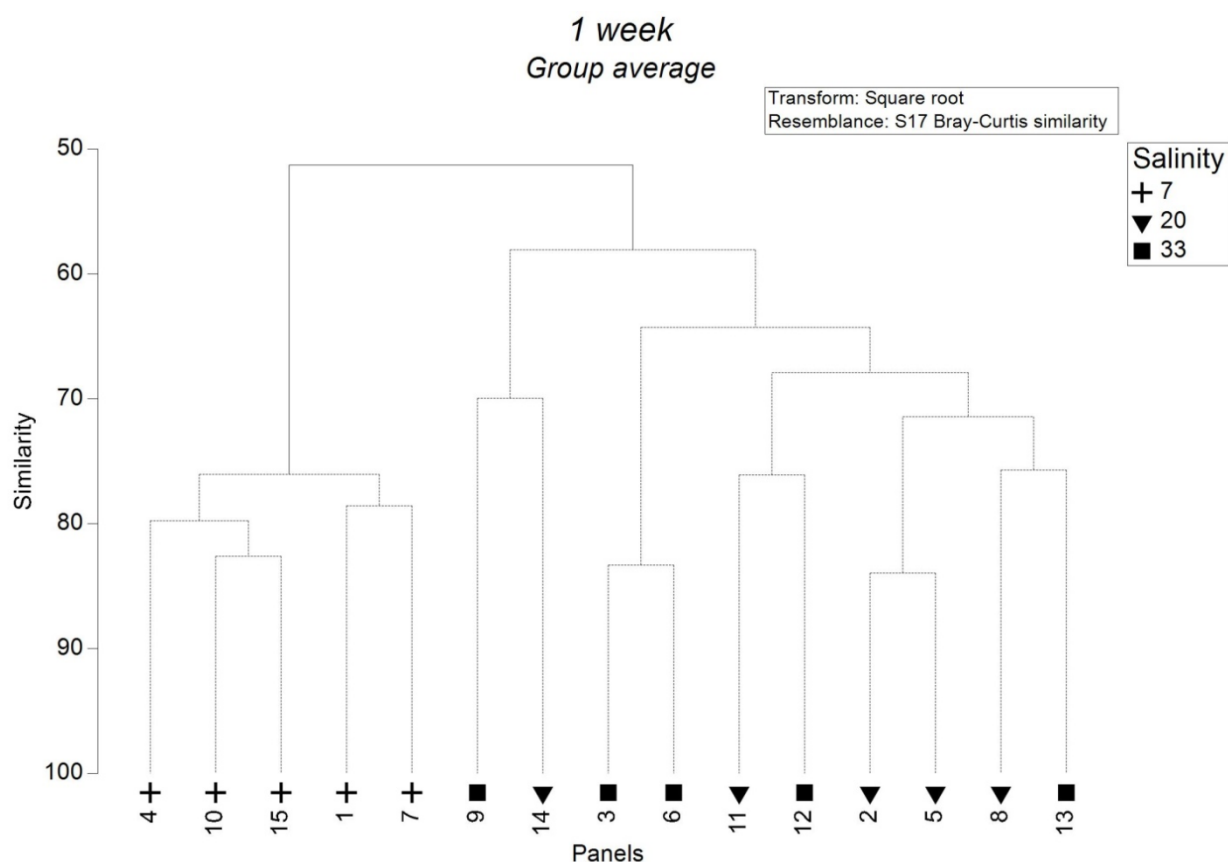


**Figure 6.4. Salinity profile during the first phase of the experiment.**

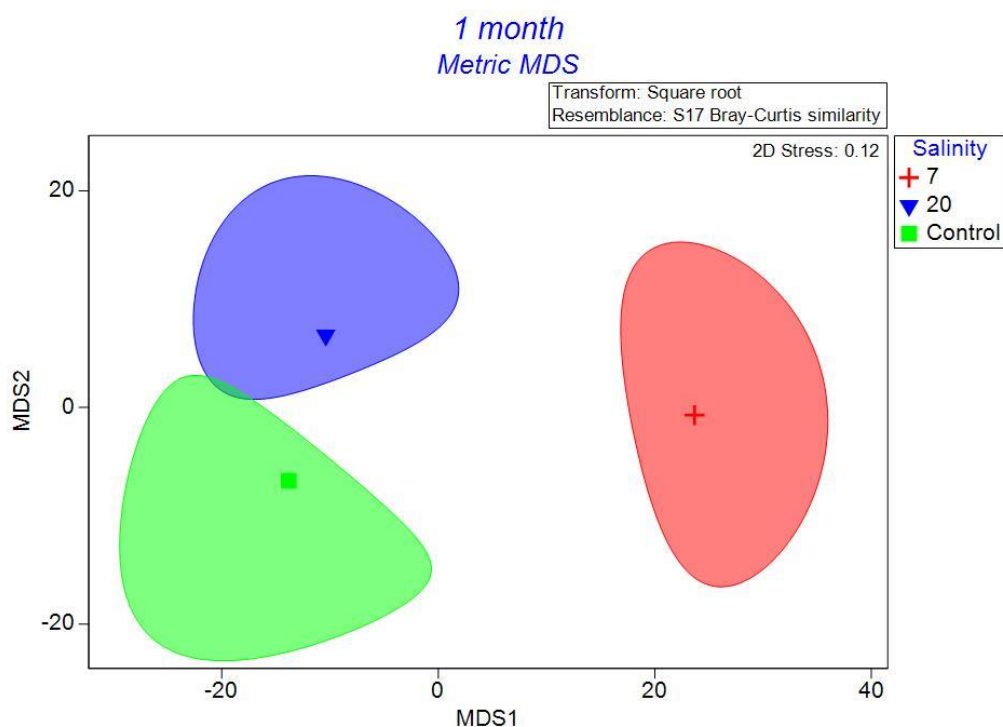
The second phase of the study looked at panel species composition, abundance and mortality. Similarities among panels exposed to different salinities (7 psu, 20 psu and 33 psu (control)) and along time (before treatment, after treatment, and one week and one month after the exposure) were analysed. The idea was to verify whether panel (assemblages) differed in their responses to different salinities over time within group and among groups.

Biofouling communities were similar on panels before and immediately after treatment but thereafter there were marked differences since low salinity treatments killed most of the organisms present. Cluster analysis of the biofouling community composition one week

after the treatment (Figure 6.5) showed that panels submitted to the same treatment were clustered together, as they had similar communities. Tight clustering was found for panels exposed to 7 psu; few mortality effects were found at 20 psu and no effects were found on control panels (33 psu) with panels exposed to these treatments clustered together. After one month (Figure 6.6) the clear separation between the two groups still evident.



**Figure 6.5. Dendrogram showing significant separation between biofouling communities grown on settlement panels treated with 7 psu and all the others treated with 20 psu and 33 psu (n=5 for each treatment).**



**Figure 6.6. Bootstrapped average regions for salinity effects one month after treatment exposure (n=5).**

On panels treated with 7 psu terebellid worms quickly disintegrated and the erect bryozoan *Bugula neritina* leached a purple / brown colour into the water. The native ascidian *Ciona intestinalis* was less reactive when touched with forceps than before the exposure. Neither *Dendrodoa grossularia*, the most frequent organisms on all panels, nor *Watersipora subatra* colonies showed immediate visual responses to the treatments. After one week levels of mortality were much more noticeable: for example 142 *D. grossularia* were counted on the five panels submitted to 7 psu - after a week 52 of these disintegrated when touched and were clearly dead. Erect bryozoans fell apart when touched with forceps and all of the *Ciona intestinalis* had fallen off the panels. All of the native ascidian *Ascidella mentula*, were killed by the 7 psu treatment and had lost colour with flaccid tests filled with a dark liquid of rotting tissue. Most organisms exposed to the 33 or 20 psu treatments survived (Figure 6.7). More grid squares with bare panel or biofilm were



counted on all panels treated with 7 psu (Table 1). All *W. subatra* individuals were dead after a week with dark slime covering the panels and the distinct odour of rotting organisms.



**Figure 6.7.** A) Settlement panel one week after exposure to a 33 psu treatment showing the high biomass and diverse biofouling community that had developed over two years at 1.5 m depth in a marina off Plymouth, UK. B) Example of a panel one week after exposure to a 20 psu treatment with many members of the biofouling community still alive. C) Panel one week after a 7 psu treatment showing black sulphurous rotting tissues. D) Typical panel appearance one month after exposure to 7 psu showing a much reduced fouling community.

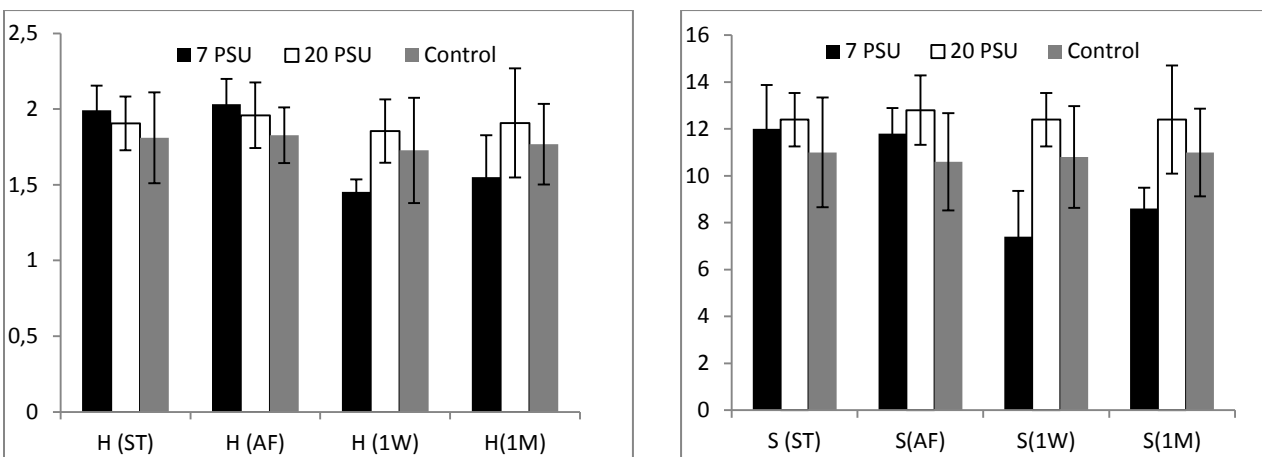
In addition, higher number of grid squares with blank and/or biofilm were counted in all panels treated with 7 psu; for blank spaces this number raised by a factor of 2 to >6.

In the 20 psu exposures *C. intestinalis* were less responsive immediately after treatment. After one week, 50% of *W. subatra* colonies were dead, of a total of 60 *D. grossularia* only two (3.3%) had died. Many *D. grossularia* individuals were covered with *Diplosoma listerianum*, not previously observed. This colonial tunicate is widespread in the United Kingdom and shows rapid reproduction and growth rates (Bullard et al., 2004, 2007; Vance et al., 2009).

One month after exposure to the three salinity treatments there were still very clear differences among the treatment groups although some recolonisation had begun on the 7 psu panels (Table 6-1). Numbers of species and Shannon-Wiener diversity index show a decrease in diversity after one week and a small increase after one month for panels exposed to 7 psu (Figure 6.8).

**Table 6-1. Average number of biofouling individuals per panel subjected to treatment with 7 psu, 20 psu and 33 psu (control) water, showing % change in abundance after one week and after one month.**

Taxa	Abundance data (average number of individuals/panel $\pm$ SD n=5) Pre treatment			% change after 1 week			% change after 1 month		
	7 psu	20 psu	Control	7 psu	20 psu	Control	7 psu	20 psu	Control
Bare substratum	8.2 $\pm$ 3.0	4.2 $\pm$ 5.3	5 $\pm$ 3.3	404.9	142.9	40.0	385.4	109.5	48.0
Biofilm	27.2 $\pm$ 11.7	28.6 $\pm$ 14	23 $\pm$ 10.4	21.3	-2.8	3.5	83.8	-15.4	13.9
<i>Sycon ciliatum</i>	0.4	3 $\pm$ 1.7	4.6 $\pm$ 6.2	0.0	-33.3	-17.4	-100.0	-80.0	-78.3
<i>Halichondria panicea</i>	3.8 $\pm$ 3.5	2 $\pm$ 1.7	7.2 $\pm$ 8	5.3	170.0	-30.6	21.1	70.0	-27.8
<i>Corynactis viridis</i>	1.6 $\pm$ 4.2	0.0	0.0	-100.0			-75.0		
Sabellariidae	2 $\pm$ 1.5	1.2 $\pm$ 0.6	0.2 $\pm$ 0.7	0.0	0.0	100.0	-100.0	-16.7	0.0
<i>Pomatoceros</i> sp.	1 $\pm$ 1.2	0.8	0.0	860.0	200.0		1060.0	325.0	
Terebellidae	0.0	0.2	0.8 $\pm$ 2.8		-100.0	-50.0		-100.0	-100.0
<i>Watersipora subatra</i>	1 $\pm$ 1.2	0.8 $\pm$ 1.4	0.4 $\pm$ 0.6	-100.0	75.0	-100.0	-20.0	225.0	-100.0
<i>Bugula neritina</i>	7.8 $\pm$ 4.6	8.6 $\pm$ 9.7	8 $\pm$ 11	-100.0	-67.4	57.5	-46.2	-51.2	42.5
erect bryozoans	12.6 $\pm$ 8.6	10.6 $\pm$ 8.3	12.6 $\pm$ 10.7	-100.0	-32.1	-23.8	-58.7	-30.2	-33.3
<i>Anomia</i> sp.	0.0	0.0	0.0						
<i>Aplidium glabrum</i>	1.6	0.0	0.0	-100.0			-25.0		
<i>Diplosoma listerianum</i>	1 $\pm$ 0.7	2.6 $\pm$ 2.1	0.6 $\pm$ 1	-20.0	-53.8	366.7	-100.0	38.5	200.0
<i>Botryllus schlosseri</i>	0.8 $\pm$ 1.4	0.0	0.0	-100.0			-100.0		
<i>Asterocarpa humilis</i>	1.0	0.6	0.6 $\pm$ 1	-100.0	66.7	-33.3	-100.0	166.7	-100.0
<i>Styela clava</i>	0.0	0.2	0.2 $\pm$ 0.7		0.0	-100.0		-100.0	-100.0
<i>Corella eumyota</i>	0.0	0.0	0.4 $\pm$ 0.6			-100.0			-100.0
<i>Clavelina lepadiformis</i>	4.6 $\pm$ 5.3	6.2 $\pm$ 6.9	8.6 $\pm$ 15.7	-100.0	-38.7	-67.4	65.2	19.4	-18.6
<i>Asciidiella aspersa</i>	7.2 $\pm$ 8.7	7.8 $\pm$ 7.3	3.8 $\pm$ 5.1	-88.9	-46.2	10.5	-100.0	-23.1	-63.2
<i>Asciidiella scabra</i>	0.0	0.0	0.0						
<i>Ascidia conchilega</i>	0.0	0.0	0.2 $\pm$ 0.7			-100.0			0.0
<i>Ascidia mentula</i>	8.4	2.8 $\pm$ 5.7	12.4 $\pm$ 43.8	-100.0	-21.4	12.9	-100.0	-100.0	8.1
<i>Ciona intestinalis</i>	18.6 $\pm$ 14.1	14.2 $\pm$ 6.9	10.4 $\pm$ 4.7	-100.0	-33.8	-15.4	-100.0	-43.7	32.7
<i>Dendrodoa grossularia</i>	29 $\pm$ 17.9	44.6 $\pm$ 17.6	43.2 $\pm$ 30.1	-37.9	19.3	9.3	-52.4	26.0	1.9



**Figure 6.8. A) Average number of species and B) Shannon-Wiener diversity index ( $H'$ ) of two year old biofouling communities developed on PVC panels at 1.5 m depth in a marina off Plymouth, UK. Panels exposed to salinities of 7, 20 and 33 psu (Control) before treatment (ST), immediately after exposure (AF), one week after (1W) and after one month (1M). Error bars are  $\pm$  SD,  $n=15$ .**

Results from one-way analysis tests for differences between salinity groups for one week and one month time after treatment points showed stronger R (Annex 3) confirming the similarity layout obtained with the cluster analysis and observed during the experiment.

Other indications of efficacy of 7 psu treatments over time are observed from mean plots of blank spaces, biofilm and *Pomatoceros* sp. *C. lepadiformis* mean plot corroborated to visual observations after one month, when the settlement of new populations was observed. *C. intestinalis* mean plot showed a significant decline of individuals after 1 week and 1 month whilst *D. grossularia* presented a relatively steady averaged number of individuals along time as relatively few effects were perceived. All plots are shown in Annex 4 and represent averages of abundance of all panels exposed to 7 psu taking into account the four recovery periods; before, immediately after, in one week and one month after exposure.

## 6.4. Discussion

We obtained a steady value of 7 psu inside our model sea-chest when immersed at Millbay marina while flushed with freshwater. This was the minimum salinity used in an experiment to assess the mortality of fouling organisms attached to PVC panels when exposed to three different salinities (7 psu, 20 psu and 33 psu (Control)). The 7 psu treatment was highly effective at killing most of the macrobenthos on the panels, whereas communities exposed to 20 and 33 psu were largely unaffected. There was some recolonization of bare substrata on the panels after one month, thus this treatment would be best carried out on sea-chests before a vessel leaves port, if she is destined for another biogeographic region.

The first phase of the experiment was set with a view to establishing the lowest achievable salinity inside our box-shaped structure built to simulate the sea-chest from a vessel. Results from this phase were fundamental to design the second phase where different salinities were used to assess the mortality of fouling organisms attached to PVC panels. We were able to reach a steady value of 7 psu inside the structure when immersed at Millbay marina (ca. 2 metre depth) and supplied with freshwater from the pontoon after >1 hour. The fact that freshwater exposure is an efficient way of controlling marine fouling organisms might be intuitive since it affects the organisms osmotic regulation and has already been successfully demonstrated in the literature (Moreira et al., 2014; Quinton, 2014; Minto, 2014; Jude & Dunphy, 2016). Achieved results from the first phase of this experiment aimed to determine an *in situ* salinity value achievable when vessels are in the port / marina, considering that a mixed salinity 'bubble' is due to be set inside the structure when the inflow of freshwater occurs. A 20 psu value (average between the minimum salinity found inside our sea-chest and the average salinity found in the marina

during the experiment) was also tested as well as controls. Minto (2014) and Quinton (2014) found intermediate effects over fouling assemblages from 24 psu and below, also supporting the use of 20 psu treatments in this study; panels were exposed to hypo salinity treatments for periods of three days though. Exposure time was based on studies conducted by Moreira et al., 2014 and Jude & Dunphy, 2016, with two hours being the minimum common period of time where 100% species mortality has occurred due to the exposure to freshwater. Notwithstanding the fact that exposure time should be as short as possible to make feasible for ships to adopt it as a routine for controlling the biofouling.

Results showed very good results for 7 psu treatments after one week of exposure although the native species (*D. grossularia*) showed only 38% of mortality while the non-species (*W. subatra*) showed 100% (Table 6-1). These results took into account the number of live and dead individuals counted on the panels. For erect bryozoans assessed randomly under the microscope, they were considered dead when colonies were pale and semi-transparent, with no shadow mass in the body cavity, whilst the bleached colour of the organisms was used as an indication of death for *B. neritina*. Deformities found in *A. mentula* and absence of *C. intestinalis* from panels reached 100% in both cases after one week and one month assessments. In that sense, mortality effect was very significant after one week except for *D. grossularia*. In this latter case, we observed a decreased number of live specimens in four of five panels treated with 7 psu within the first week. After one month, the decline of specimens was huge, except in one panel as observed for the first week evaluation, however even in this case the number of species decreased.

After one week many dead specimens / rotting organisms were seen in the panels, while after one month few were observed. A possible reason for that may be the decreased number of species within the first week and the huge decrease after one month, probably indicating species detachment from panels (those affected by the treatment) within the period of recovery. An increasing number of blank and biofilm spaces, along with the

increase in *Pomatoceros* sp (probably covered by other species and not initially detected) and the complete absence of *C. intestinalis* were also considered very good indications of the efficacy of 7 psu treatments (Table 6-1)). One week and one month results thus showed a general decrease in the abundance species and diversity of fouling organisms (Figure 6.8). At the same time, after one month, new populations of *Clavelina lepadiformis* were observed (Annex 4), as well as small turfs of erect bryozoan and new colonies of *W. subatra*. Recruitment was probably facilitated by the season (summer) the experiment occurred and the availability of unfouled substrate after the demise of many organisms. Of the two commonest species found in this study, *D. grossularia* and *C. intestinalis*, the first is a small, robust tunicate, while the second is large, soft and highly contractile tunicate. Their bauplan possibly contributed to their differing vulnerability to the treatment. One-way analysis of similarity (ANOSIM tests) found an almost complete separation between samples treated with 7 psu and those kept in control ( $R = 0.96$ ) and a strong  $R$  for the pair 7 psu - 20 psu ( $R = 0.75$ ) after one week. After one month however, the global  $R$  decreased as well as the  $R$  for the pair 7 psu - control but still strong and significant ( $R = 0.74$ ). These results indicate that the treatment is very effective but it is time dependant, new species were recruited for the increased blank / biofilm spaces left after the first week. For vessels which stay longer periods in berth it may represent a decrease in the efficiency of hyposaline treatments; suggesting that this kind of treatment should always be applied before vessels departing for the next port of call. ANOSIM tests didn't show significant differences between 20 psu treatments and control ones in any cases (All results obtained with the ANOSIM tests are presented in Annex 3).

## 6.5. Conclusion

Very high levels of mortality occurred in mature biofouling communities subjected to two hour treatment with 7 psu water, although some *Dendrodoa grossularia* were resilient. Low

salinity treatments can be an efficient way of minimizing biofouling from ship sea-chests, and offer a promising tool to be incorporated in vessel operation. This would be an environmentally friendly biosecurity tool for minimizing and controlling ships sea-chest biofouling that is simple and would not cause undue delay or costs. Limitations of this study are related to its representativeness of one single geographic area, to the fact that the composition of the fouling community can be highly diversified with some organisms being more adjustable to unfavourable conditions than others and yet to the static conditions faced by the organisms during their development in the marina which differ from a ship sea-chest en route.



## **Chapter 7 : General discussion**

## **7.1. The Ballast Water Management Convention (IMO, 2004)**

The role of shipping as an unintentional and efficient pathway for spreading aquatic non-native species around the globe lead to the adoption and implementation of international standards to control and minimise this transfer, i.e. the International Maritime Organization Ballast Water Management Convention (IMO, 2004). At the same time, it has fostered the necessity of developing techniques to comply with these rules by means of ballast water treatment systems able to reduce to a minimum the concentration of viable organisms in ballast tanks. Portable instruments developed to verify the efficacy of these systems are also a key component of the compliance, monitoring and enforcement measures to fulfill with international regulations on ballast water.

As shown in previous chapters, the Ballast Water Management Convention entered into force in September, 2017. However, unlikely the majority of Conventions adopted under the scope of the International Maritime Organization, the Ballast Water Management Convention requires ships to comply with requirements that are beyond the usual certificates, record books or surveys. In the present case, ships need to comply with what is called the performance or biological standard; which means that there is a maximum concentration of viable organisms according to the size or group allowed in ballast water discharges. This performance standard is a novelty and requires both ships and port State inspectors to be familiarised with new protocols and procedures.

All ships registered under contracting Parties to the Ballast Water Convention, which take up, use and discharge ballast water during international voyages must comply with regulations and can be enforced by coastal States. The obligation of complying with the performance standard based on numbers of organisms per unit of volume has brought up the necessity of researching and developing methods able to reduce to a minimum the number of organisms in ships ballast water. As a result, ballast water treatment systems

were proposed as a way of managing ships ballast water in such a way that virtually all organisms are likely to be non-viable when ships discharge their ballast water. Most systems use mechanical tools (e.g. filters) and physical or chemical components to treat the ballast water. When systems make use of active substances for treating the ballast water, they must comply with a two-step approval process conducted through the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection – Ballast Water Working Group (Regulation D-3, paragraph 2). For all systems however, there is a need to apply for a Type Approval Certificate issued by the Administration (Regulation D-3, paragraph 1).

From the perspective of the inspection regime, and in order to verify ships compliance to the performance standard, regulation D-2, port State control officers will be responsible to enforce the regulations in place. Nevertheless, it is worth noticing that in the majority of cases, inspections will rely mainly on documents, with few cases progressing to sampling procedures according to the four-stage inspection process defined by the Marine Environment Protection Committee - Resolution MEPC Res.252(67).

In order to support the initial phase of transition associated with the implementation of a new regulation which involves novel concepts and requirements, it was initially agreed at the IMO, a trial period of two years for gathering data and to help designing a more homogenous enforcement in terms of sampling and analysis protocols. During this period, port States should refrain from applying criminal sanctions or detaining the ship, based on sampling; however this does not prevent them from taking preventive measures to protect its environment, human health, property or resources (BWM.2/Circ.42). Specifically for the port State control regimes, it gives them time to adjust procedures internally and share best practices among them and through the Implementation of IMO Instruments Sub-Committee for instance.

Aspects like the trial phase and others associated were discussed by the IMO during the 71<sup>st</sup> session of the Maritime Environmental Protection Committee, in July 2017. Resolution MEPC.290(71) was adopted to address the experience-building phase associated with the Ballast Water Convention with a view to allowing port States, flag States and stakeholders to gather and analyse data, and based on these data propose amendments to the Treaty. This phase has begun with the coming into force of the Convention and will end with the entry into force of the priority amendments, which may mean a relatively long time; certainly few more years than the initial trial period agreed.

## **7.2. Discussion of the main findings and implications for the shipping industry**

The necessity of having new expensive systems on board was not initially welcome by shipping owners. The fact that no suitable technologies of treatment were available by the time the Convention was adopted had possibly resulted in an additional obstacle to have it ratified by Member-States and contributed to the delay of thirteen years between its adoption and enforcement. More specifically, shipping owners called upon feasibility aspects considering that commercial ships usually have small engine rooms; so how to fit these new expensive systems in ships already in use? On the other hand, manufacturers and entrepreneurs have seen it as a challenge and an opportunity. It's worth noticing that poor availability of ballast water systems are no longer an obstacle and currently at least 75 ballast water management systems have already received the Type Approval Certification by the Administration (as updated in May, 2018): <http://www.imo.org/en/OurWork/Environment/BallastWaterManagement/Documents/Table%20of%20BA%20FA%20TA%20updated%20May%202018.pdf>.

As seen in chapter 2, until 2015, 44 non-native species that have become invasive with high ecological impacts in the North-eastern Atlantic and 16 in the South-western Atlantic were described in available literature and databases. The main cited pathway of introduction and / or spread of these invasive species was shipping, both ballast water and biofouling, with circa of 65 % of the records. Chapter 2 reinforces the role of the shipping as a main pathway of introduction and spread of invasive species recorded in both North-eastern and South-western Atlantic Ocean, however no species recorded as invasive could be directly related to the shipping trade between the two regions. There is still a need to investigate the main vectors of introduction and spread and to address them accordingly since they usually work in a complementary way depending on species life stages and on the interplay of environmental factors. In the present case, it means the necessity of having biofouling under control together with ballast water.

As already mentioned the Ballast Water Convention allows a maximum concentration of 10 viable cells larger or equal to 10  $\mu\text{m}$  and smaller than 50  $\mu\text{m}$  per millilitre in ballast water discharges. Having that, fractionation methods are commonly used to separate the organisms in this size range. As discussed in Chapter 5, traditional size fractionation methods that use nylon mesh filtration of 10  $\mu\text{m}$  mesh showed considerable size bias, resulting in an overestimate of the number of cells per millilitre in the size class defined by the Convention for phytoplankton organisms. Since many fluorometers used in the quantification of the IMO relevant size class of 10 – 50  $\mu\text{m}$  include a filtration step, the bias due to smaller size cells retained on the filter may be significant. Results also showed much higher chlorophyll values than measured with the flowcytometer that may affect semi-quantification methods that use chlorophyll as proxy for cell density.

Another aspect to be considered is how these devices would respond to a large number of smaller cells (smaller than 10  $\mu\text{m}$ ) considering that this fraction corresponds to more

than 95 % of the total cells in the size range between 2 and 50  $\mu\text{m}$ . Would their fluorescence signal influence the numeric results achieved with the fluorometers? It is not clear.

From the perspective of shipping owners it would be like investing a great amount of money to buy a system that could fail to pass port State control inspections due to false positives resulting from the instrument used for assessing compliance. Abundance results should be read together with the fluorescence measurements provided by the portable fluorometers, and yet these results are intended to be indicative of risk. These aspects should be clear for the inspection team. Moreover, although regulations specify numbers, rely only on abundance results (number of cells / millilitre), in any stage of inspection, except in the fourth stage or when gross non-compliance is evident, might lead to misinterpretations and consequently to legal uncertainties.

The pattern of distribution of viable and non-viable cells was investigated over one year in a natural assembly (L4) and in treated ballast water samples (Chapter 4) since together with the concentration of cells per millilitre, cells viability is also in check by the D-2 standard. Different techniques based on fluorescence were used and compared: two portable fluorometers developed to measure concentrations of phytoplankton cells in ballast water, flow cytometry and vital staining microscopy methods. Samples were collected in the English Channel over one year and during ballast water shipboard efficacy tests (Figure 7.1). Despite the fact that the fluorometers were developed for lower concentration of cells and, therefore, accurate results for higher concentrations of cells in the sample are not necessarily expected, a moderate / relatively strong correlation coefficient with the flow cytometry was found. In addition, fluorescence results from both fluorometers were highly concordant as seen in previous studies (Gollasch et al., 2012; Bradie et al., 2017).



**Figure 7.1. Testing treated ballast water samples with two portable fluorometers (Ballast Check 2 and FastBallast) during a ballast water shipboard efficacy test in Southampton, UK.**

Analysis of treated ballast water samples showed a large variation in the number of viable cells and among replicates, however indicating the same level of risk on all occasions for regulatory purposes. One key aspect to bear in mind is on the possible benefits and limitations of each technique when sampling for compliance, monitoring and enforcement. Many and varied technologies of treatment are currently available and no excuses might be used to postpone the use of ballast water management systems on board ships. However, potential sources of error suggest that portable devices developed for supporting inspection regimes may need further refinement. On the other hand and from the inspection perspective, the use of fluorescence for indicative analysis is fast, simple and represents a reliable method, allowing the inspection to be conducted by port State control officers until the third stage of inspection. The last stage certainly demands more

expertise, when a support team of specialists is necessary to carry it on, it also demands more time and it is certainly more costly.

Eventually, stakeholders involved in the process should be aware that all techniques have limitations opting for the best and more convenient ones to address their needs.

Chapter 3 of this thesis presents a case of study of a unilateral standard of ballast water adopted and enforced by Brazil in her national waters. Results from the ten first years of implementation suggest that the level of compliance with the regulation increased along the years. The Ballast Water Convention entered into force on 8<sup>th</sup> September 2017, and even though thirteen years have passed from its adoption until its entry into force, it seems that the maritime community is not completely aware and able to implement its requirements as well as to enforce them. Taking into account the case of study conducted in Brazil, it is expected that after the initial period of implementation, things will settle down and goes smoothly. In light of the experience-building phase adopted in July 2017, it seems that this initial transitional period has already being addressed by the Organization.

With regards to the biofouling vector, experiments were conducted to report on the efficiency of using low salinity as a treatment to kill fouling organisms in areas of the ship where it is difficult to coat and therefore tend to accumulate fouling organisms, like ships sea-chest. Chapter 6 discusses a simple preventive method which is not supposed to be a solution but a step forward to improving biosecurity having the importance of the biofouling vector on the spreading of aquatic non-native species. Experiments conducted in a marina in Plymouth (Figures 7.2 and 7.3) with panels with well-developed macrofouling communities showed that short periods of exposure to low salinity were able to kill the vast majority of macrobenthos. Simple approaches like this one can be included in ships routine before departing to the next port of call. Similarly, it can be easily implemented in recreational vessels which commonly stay long in berth and always before departure, since the treatment is time dependant as shown in the chapter.

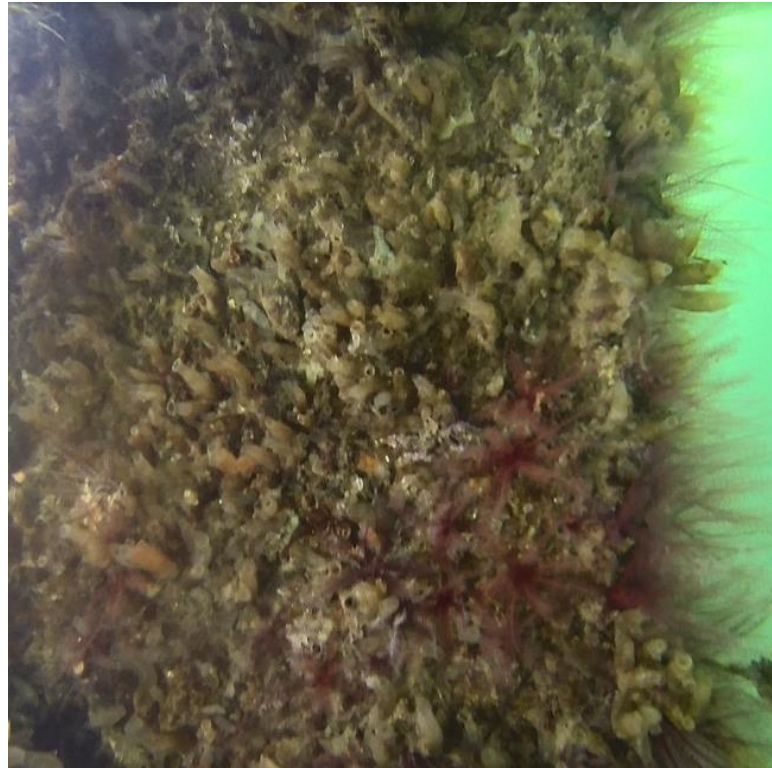


The IMO Guidelines on biofouling (Resolution MEPC.207(62)) discuss the implementation of practices for managing biofouling, with a view to minimizing the transfer non-native species and indirectly improving ships energy efficiency, another important issue dealt by the IMO. In that sense, the use of low salinity treatments to manage biofouling meets and collaborates with the concerns expressed by the voluntary guidelines. As shown, results can be very effective at killing the majority of fouling species, and in areas where is normally difficult to use anti-fouling coatings.

With the approval of the new IMO Project “GloFouling” to address ships biofouling, in May 2017, the idea that soon the biofouling guidelines will be replaced by an international Treaty has grown stronger. Therefore, the adoption of simple and effective measures as the one proposed in chapter 6 works proactively in favour of the implementation whether there are guidelines or an international convention in place. After all, the utter idea is to minimise the threat posed by invasive aquatic species through shipping. Moreover, the adoption of international regulations sooner than latter is good for the environment, avoids legal uncertainties and helps preventing scepticism in the shipping industry once again.



**Figure 7.2. Fouled panels being collected / returned during the experiment with low salinity in a Marina in Plymouth, UK.**



**Figure 7.3. Pontoon colonised by fouling organisms in the marina where the biofouling experiment was conducted (Plymouth, UK).**

### **7.3. Conclusions**

Some important findings resulting from this Ph.D are listed hereafter and may deserve further investigation and / or implementation:

- An important aspect which was not an objective of this thesis pertains to the importance of the aquaculture as a pathway for the transfer of non-native species. Results from the review conducted in this research suggest this pathway is nearly as important as shipping in the North-eastern Atlantic Ocean;
- Enforcement procedures adopted by the administration, i.e. maritime / port authorities, must be monitored with a view to assessing compliance of ships and depending on the results provoke the implementation of new or adjust policies for inspection. After the initial phase of transition, procedures should become a smooth

and straightforward experience and will be part of the routine of ships and inspection regimes;

- Fluorometers tend to overestimate chlorophyll biomass in samples of natural assemblies of phytoplankton as a result of a filtration step usually required by these instruments causing a significant bias due to size cells retained on the filter. This should be taking into account considering the different scenarios to be faced by ships in different regions of the world. Notwithstanding, the two fluorometers used in this research were developed to provide indicative results of risk for ballast water samples where low concentrations of organisms are expected. Thus, accurate results for higher concentration of cells are not necessarily expected;
- In an ocean susceptible to a variety of natural and anthropogenic factors, phytoplankton size composition tends to be affected as well as the cell community structure and the chlorophyll biomass. This may pose additional challenges for indicative tools developed to measure abundance of photoautotrophic cells in the water;
- All fluorescence methods available for measuring phytoplankton viability have limitations. At the same time, high-end standard methods like flow cytometry showed a possible over-estimation of cells whenever the number of non-viable however intact cells were present in the sample. The latter scenario is highly probable in UV treated ballast water samples. In this case, proper calibration is essential;
- Fluorescence chlorophyll measured with hand-held equipment tended to co-vary strongly; with regards to the results obtained with flow cytometry moderate / relatively strong correlation coefficients were found;
- A huge variation among replicates and methods implemented to analyse ballast water samples in this research appear to indicate that methods need refinement.

However, as long as the benefits and limitations are known, any of them could be used to indicate risk;

- Hyposalinity treatments should be taken as promising treatments for killing macrofouling organisms and can be suggested as a simple, low-cost tool for controlling biofouling in ships sea-chest. In this regard, flush sea-chests with freshwater for at least two hours before leaving the port for a different biogeographic area can represent an important step forward in improving biosecurity; and
- In most cases, it is difficult to disentangle the level of influence of the different vectors and / or pathways in the transfer of non-native species, and a combination of vectors may carry species at multiple life stages. As a result, a general approach to control / minimise the threat represented by the unintentional transfer of non-native species should consider the adoption of integrated studies. Important vectors should be considered together as the best way to move onward, with ballast water representing one big step forward.

## **Annex 1**

### **Table 2-2 (chapter 2): Invasive marine and oligohaline species recorded for the NE Atlantic Ocean**

**Table 2-2: Invasive marine and oligohaline alien species recorded for the NE Atlantic Ocean.**

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Dinoflagellata</b>	<i>Alexandrium monilatum</i>	Eastern Pacific and western Atlantic Oceans.	Produces fish mortality-causing and haemolytic compounds (harmful effects)	Fraga, S.; Moestrup, Ø. /WoRMS; Indian River Lagoon Species Inventory (www.sms.si.edu/irlspec)	Shipping	Balech, E. 1995. The genus <i>Alexandrium</i> Halim (Dinoflagellata). Sherkin Island Marine Station, Cork, Ireland. 151pp. Hallegraeff, GM. 2003. Taxonomic Principles. In: Hallegraeff, GM, Anderson, DM & AD Cembella (Eds.). 383-432. Manual on Harmful Marine Microalgae. UNESCO Publishing, Paris. 793pp. www.sms.si.edu/irlspec/index.htm Katsanevakis, S.; Bogucarskis, K.; Gatto, F.; Vandekerkhove, J.; Deriu, I.; Cardoso A.S. (2012). Building the European Alien Species Information Network (EASIN): a novel approach for the exploration of distributed alien species data. BioInvasions Records. 1: 235-245.
<b>Dinoflagellata</b>	<i>Karenia mikimotoi</i>	Japan	Fish and invertebrate mortality; This species is a recurring bloom former in coastal waters of Japan and Korea; red tides commonly occur in warmer months and are associated with massive fish and shellfish kills.	WoRMS; Aleksas Naršcius/AquaNIS	Shipping	Gollash, S.; Nehring, S. (2006). National checklist for aquatic alien species in Germany. Aquatic invasions 1(4): 245-269 Satake, M., Shoji, M., Oshima, Y., Naoki, H., Fujita, T. & Yasumoto, T. (2002). Gymnocin-A, a cytotoxic polyether from the noxious red tide dinoflagellate, <i>Gymnodinium mikimotoi</i> . Tetrahedr. Lett. 43: 5829-5832. Hallegraeff, G.M., Anderson, D.M. & Cembella, A.D., Eds (2003). Manual on harmful marine microalgae. Paris: UNESCO. Katsanevakis, S.; Bogucarskis, K.; Gatto, F.; Vandekerkhove, J.; Deriu, I.; Cardoso A.S. (2012). Building the European Alien Species Information Network (EASIN): a novel approach for the exploration of distributed alien species data. BioInvasions Records. 1: 235-245. Satake, M., Tanaka, Y., Ishikura, Y., Oshima, Y., Naoki, H., Yasumoto, T. (2005). Gymnocin-B with the largest contiguous polyether rings from the red tide dinoflagellate, <i>Karenia</i> (formerly <i>Gymnodinium</i> ) <i>mikimotoi</i> . Tetrahedron Lett. 46, 3537-3540.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Bacillariophyceae</b>	<i>Odontella sinensis</i>	Hong Kong, Red Sea, coastal waters of India, Gulf of Siam, Yellow Sea and the East Chinese Sea	Even during blooms, other native species occur in higher numbers. However, long-term investigations have shown that the population growth of other phytoplankton species was depressed by high populations of this species.	Stephan Gollasch/DAISIE	Ballast water	Boalch GT, Harbour DS (1977) Unusual diatom off the coast of south-west England and its effect on fishing. <i>Nature</i> 269:687-688 Ostenfeld CH (1908) On the immigration of <i>Biddulphia sinensis</i> Grev. and its occurrence in the North Sea during 1903-1907. <i>Meddelelser fra Kommissionen for Havundersogelser, Plankton</i> 1(6):1-25 Wallentinus I (2006) Introductions and Transfers of plants. In Annex 8: Status of Introductions of Non-indigenous Marine Species to North Atlantic and Adjacent Waters According to National Reports Considered at Meetings of the Working Group on Introductions and Transfers of Marine Organisms 1992-2002 of the Report of the Working Group of Introductions and Transfers of Marine Organisms, Oostend, Belgium, 16-17 March 2006. ICES CM 2006/ACME:05, pp 150-221 [available at <a href="http://www.ices.dk">www.ices.dk</a> ]
<b>Ochrophyta</b>	<i>Undaria pinnatifida</i>	Northern Asia	Can be dominant during mass events and causes losses to aquaculture	Stephan Gollasch/DAISIE; NOBANIS	Unintentional introduction with oyster; Probable secondary dispersal caused by vessels.	Boudouresque CFM, Gerbal M, Knoepffler-Peguy M (1985) L'algue japonnaise <i>Undaria pinnatifida</i> (Phaeophyceae, Laminariales) en Méditerranée. <i>Phycologia</i> 24:364-366 Floc'h J-Y, Pajot R, Wallentinus I (1991) The Japanese brown alga <i>Undaria pinnatifida</i> on the coast of France and its possible establishment in European waters. <i>Journal of the Conseil International pour l'Exploration de la Mer</i> 47: 379-390 Wallentinus I (2006) Alien Species Alert: on <i>Undaria pinnatifida</i> (Wakame or Japanese kelp). Annex 9. In Gollasch S, Kieser D (eds) Report of the Working Group of Introductions and Transfers of Marine Organisms, Oostend, Belgium, 16-17 March 2006. ICES CM 2006/ACME:05, pp 292-327 [available at <a href="http://www.ices.dk">www.ices.dk</a> ] Irigoyen, A.J., Trobbiani, G., Sgarlatta, M.P., Raffo, M.P. (2011). Effects of the alien algae <i>Undaria pinnatifida</i> (Phaeophyceae, Laminariales) on the diversity and abundance of benthic macrofauna in Golfo Nuevo (Patagonia, Argentina): potential implications on local food webs. <i>Biol Invasions</i> (2011) 13:1521–1532.



Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Ochrophyta</b>	<i>Sargassum muticum</i>	NW Pacific	Outcompetes native species for resources and/or space	Anna Occhipinti/AquaNIS; Guiry, Michael D.2015). In: Guiry, M.D. & Guiry, G.M. (2015). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway/WoRMS	Biofouling, aquaculture	Yendo K (1907) The Fucaceae of Japan. Journal of the College of Science, Tokyo Imperial University 21 (Article 12): 102-106, plate XV, fig. 1-4. Critchley AT, Farnham WF, Yoshida T, Norton TA (1990) A bibliography of the invasive alga <i>Sargassum muticum</i> (Yendo) Fensholt (Fucales; Sargassaceae). Botanica marina 33: 551-562 Wallentinus I (1999) Exotics across the ocean. University of Kiel, Berlin.
<b>Rhodophyta</b>	<i>Asparagopsis armata</i>	Australia	Outcompetes native species for resources and/or space	Anna Occhipinti/AquaNIS; Guiry, Guiry, Michael D. 2015. In: Guiry, M.D. & Guiry, G.M. (2015). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway/WoRMS	Shipping	Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. Frontiers in Ecology and the Environment. 6(9): 485-492. Womersley, H. B. S. 1996. The Marine Benthic Flora of Southern Australia. Rhodophyta. Part IIIB. Gracilariales, Rhodymeniales, Corallinales and Bonnemaisoniales. Australian Biological Resources Study & State Herbarium of South Australia, Canberra, 392 pp. Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. Aquatic Invasions. 8(1): 3-19.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Rhodophyta	<i>Bonnemaisonia hamifera</i>	NW Pacific	It may become the dominant alga in certain regions competing with other algae and seagrasses.	Stephan Gollasch/DAISIE	Unintentional introduced with shellfish or in the hull fouling of vessels; secondary spread by drift with water currents or attached to floating objects	Farnham WF (1980) Studies on aliens in the marine flora of southern England. In: Price JH, Irvine DEG, Farnham WF (eds) The shore environment, vol 2: ecosystems. London, Academic Press. (Systematics Association Special Volume, No. 17B.), pp 875-914 South GR, Tittley I (1986) A checklist and distributional index of the benthic marine algae of the North Atlantic Ocean. St. Andrews & London, Huntsman Marine Laboratory & British Museum (Natural History) p 213 Wallentinus I (2006) Alien Species Alert: on <i>Undaria pinnatifida</i> (Wakame or Japanese kelp). Annex 9. In: Report of the Working Group of Introductions and Transfers of Marine Organisms, Oostend, Belgium, 16-17 March 2006. ICES CM 2006/ACME:05, pp 292-327.
Rhodophyta	<i>Grateloupia turuturu</i>	Eastern seas of Russia, China, Japan and Korea	It can outcompete native seaweeds in the low intertidal and shallow subtidal. It can also alter typical trophic patterns and cause loss of habitat.	CABI. Invasive Species Compendium. Wallingford, UK: CABI International <a href="http://www.cabi.org/isc">www.cabi.org/isc</a> .	Aquaculture, ballast water, hull fouling	Cabioch J, Castric-Fey A, L'Hardy-Halos MT, Rio A, 1997. <i>Grateloupia doryphora</i> et <i>Grateloupia filicina</i> var. <i>luxurians</i> (Rhodophyta, Halymeniaceae) sur les côtes de la Bretagne.) Cryptogamie Algologie, 18:117-137. Ribera A, Boudouresque CF, 1995. Introduced marine plants, with special reference to macroalgae: mechanisms and impact. In: Progress in Phycological Research, Vol. 11 [ed. by Round, F. E.\Chapman, D. J.]. Amsterdam: Biopress Ltd., 187-268. Marston M, Villalard-Bohnsack M, 2002. Molecular variability and potential sources of <i>Grateloupia doryphora</i> (Halymeniaceae), an invasive species in Rhode Island waters (USA). Journal of Phycology, 38:649-658. Farnham WF, 1978. Introduction of marine algae into the Solent, with special reference to the genus <i>Grateloupia</i> . Portsmouth, UK: Portsmouth Polytechnic, unpaginated. Farnham WF, 1980. Studies on aliens in the marine flora of southern England. In: The Shore Environment. Volume 2: Ecosystems [ed. by Price, J. H.\Irvine, D. E. G.\Farnham, W. F.]. London, UK: Academic Press, 875-914.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Chlorophyta	<i>Codium fragile fragile</i>	Japan	It alters benthic communities and habitats; It causes a nuisance to humans when it is swept ashore and rots; It fouls shellfish beds, smothering mussels and scallops, clogging scallop dredges, and interfering with harvesting. It fouls fishing nets, wharf pilings and jetties.	B. S. Galil/DAISIE	The vector to Europe is unknown; Secondary dispersal: mariculture, transport on ship hulls and net fouling.	Provan J, Murphy S, Maggs CA (2005) Tracking the invasive history of the green alga <i>Codium fragile ssp. tomentosoides</i> . Molecular Ecology 14(1):189-194 Silva, P.C. (1955). The dichotomous species of <i>Codium</i> in Britain. Journal of the Marine Biological Association of the United Kingdom 34: 565-577 Trowbridge CD (1998) Ecology of the green macroalga <i>Codium fragile</i> (Suringar) Hariot 1889: invasive and non-invasive subspecies. Oceanography and Marine Biology Annual Review 36:1-64
Chlorophyta	<i>Ulva australis</i>	Pacific Ocean	Oportunistic species, highly competitive and tolerant to a wide range of environmental conditions.	Guiry, Michael D. 2015. In: Guiry, M.D. & Guiry, G.M. (2015). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway/WoRMS; European Alien Species Information Network; CABI. Invasive Species Compendium. Wallingford , UK: CABI International <a href="http://www.cabi.org/isc">www.cabi.org/isc</a> .	Aquaculture, shipping	Katsanevakis, S.; Bogucarskis, K.; Gatto, F.; Vandekerkhove, J.; Deriu, I.; Cardoso A.S. (2012). Building the European Alien Species Information Network (EASIN): a novel approach for the exploration of distributed alien species data. BioInvasions Records. 1: 235-245. <i>Ulva pertusa</i> [Javier Cremades Ugarte]. In: Invasive Species Compendium. Wallingford, UK: CAB International. <a href="http://www.cabi.org/isc">www.cabi.org/isc</a> .

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Tracheophyta	<i>Spartina townsendii</i> var. <i>Anglica</i>	Southern England	Adverse habitat modification	IUCN's ISSG (Invasive Species Specialist Group). Global Invasive Species Database (GISD); WoRMS; VLIZ Alien Species Consortium; WoRMS (2014). <i>Spartina townsendii</i> var. <i>anglica</i> C.E. Hubbard. In: Costello, M.J.; Bouchet, P.; Boxshall, G.; Arvantis, C.; Appeltans, W. (2014) European Register of Marine Species	Widely used as a plant for stabilising mud and for land accretion to protect coastlines and prevent erosion. Its seeds may float to new localities and may also be dispersed by birds. From NOBANIS: it appears that the North American smooth cordgrass ( <i>Spartina alterniflora</i> ) was originally introduced by ballast water to Great Britain prior to 1870 (Eno et al. 1997).	Verloove, F. (2006). Catalogue of neophytes in Belgium (1800-2005). Scripta Botanica Belgica. 39. National Botanic Garden of Belgium: Meise. ISBN 90-72619-71-4. 89 pp. Eno, N.C., Clark, R.A. and Sanderson, W.G. 1997. Non-native marine species in British waters: a review and directory. - Joint Nature Conservation Committee, Peterborough: 152 pp. Hubbard, J. C. E.; Stebbings, R. E. (1967). Distribution, dates of origin and acreage of <i>Spartina townsendii</i> (sl) marshes in Great Britain. In Proceedings of the Botanical Society of the British Isles. (Vol. 7, pp. 1-7). Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. Aquatic Invasions. 8(1): 3-19.
Tracheophyta	<i>Spartina alterniflora</i>	North America	Considering it an established non native species to South America, its presence has been associated to reduction in native biodiversity (Brazil); Adverse habitat modification.	IUCN's ISSG (Invasive Species Specialist Group). Global Invasive Species Database (GISD); WoRMS	Shipping	Bortolus A, Carlton JT, Schwindt E. 2015. Reimagining South American coasts: unveiling the hidden invasion history of an iconic ecological engineer. Diversity and Distributions. Daehler, C. C., and D. R. Strong. 1996. Status, Predictions, and prevention of introduced cordgrass <i>Spartina spp.</i> Invasions in Pacific Estuaries, USA. Biological Conservation 78 (1996) 51-58. Katsanevakis, S.; Bogucarskis, K.; Gatto, F.; Vandekerckhove, J.; Deriu, I.; Cardoso A.S. (2012). Building the European Alien Species Information Network (EASIN): a novel approach for the exploration of distributed alien species data. BioInvasions Records. 1: 235-245. Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. Frontiers in Ecology and the Environment. 6(9): 485-492.

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Annelida	<i>Ficopomatus enigmaticus</i>	Unknown	Ecosystem impact: can form extensive reefs which may provide refuge for invertebrates that may have an impact on native species; Economic impact: able to build up dense tube colonies that attach to pipes cause blockages. They also foul surfaces in aquaculture ponds, ports and docks. Fouls the hulls of recreational craft and floating structures. Areas with thermal effluents may develop large colonies.	Dan Minchin/DAISIE	Probably introduced as hull fouling or as larvae in ballast water; spread with leisure craft hulls.	Davies BR, Stuart V, & Villiers M de (1989) The filtration activity of a serpulid polychaete population ( <i>Ficopomatus enigmaticus</i> (Fauvel)) and its effects on water quality in a coastal marina. Estuarine, Coastal and Shelf Science 29:613-620 Dixon DR (1981) The reproductive biology of the serpulid <i>Ficopomatus enigmaticus</i> (Mercierella) enigmaticus in the Thames Estuary, SE England. J Mar Biol Ass UK 61:805-815 Thomas NS, Thorp CH (1994) Cyclical changes in the fauna associated with tube aggregates of <i>Ficopomatus enigmaticus</i> (Fauvel). Memoires de Museum National d'Histoire Naturelle 162:575-584

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Annelida	<i>Marenzelleria neglecta</i>	Atlantic coast of the North-America.	Ecosystem impact: competes with native macrofauna for food and space. Being numerically dominant it can change the structure of a native benthic community. Burrowing activity of this worm has a high impact on fluid-exchange rates between bottom water and sediments.	Sergej Olenin/DAISIE	Ballast water	Daunys D, Zettler ML, Gollasch S (1999) <i>Marenzelleria cf. viridis</i> (Verrill, 1873). In: Gollasch S, Minchin D, Rosenthal H, Voigt M (eds) Case histories on introduced species: their general biology, distribution, range expansion and impact. Berlin, Logos-Verlag, pp 61-67 Kotta J, Orav H, Sandberg-Kilpi E (2001) Ecological consequence of the introduction of the polychaete <i>Marenzelleria viridis</i> into a shallow water biotope of the northern Baltic Sea. J Sea Res 46: 273-280 . Zettler M, Daunys D, Kotta J & Bick A. (2002). History and Success of an Invasion into the Baltic Sea: the Polychaete <i>Marenzelleria cf. viridis</i> , Development and Strategies. In: Leppäkoski E, Gollasch S & S. Olenin (eds). Invasive aquatic species of Europe – distribution, impacts and management. Kluwer Academic Publ., Dordrecht, Boston, London, pp 66-77
Arthropoda	<i>Austrominius modestus</i>	Pacific Ocean	Ecosystem impact: <i>E. modestus</i> is a bioconstructor, displaces gradually native species competing for space.	Anna Occhipinti / AquaNIS	Larvae are transported by ballast waters and adults as biofouling on ship hulls	Crisp DJ (1958) The Spread of Elminius Modestus Darwin In North-West Europe. Journal of the Marine Biological Association of the United Kingdom, 37: 483-520
Arthropoda	<i>Callinectes sapidus</i>	Atlantic Coast of USA, Caribbean Sea, North and East Coast of Brazil	Can act as a top predator, keystone species in saltmarshes. In some areas this species is associated to lose in aquaculture/commercial/recreational harvest or gain.	Anna Occhipinti / AquaNIS; WoRMS	Ballast water; Possible pathways of introduction include active migration, larval dispersal via water currents or ships's hulls.	Jorge L, Gutiérrez, Clive G, Jones, David L, Strayer, Oscar O, Iribarne. Mollusks as ecosystem. Engineers: the role of shell production in aquatic habitats. MacDonald, J. A., Roudez, R., Glover, T., & Weis, J. S. (2007). The invasive green crab and Japanese shore crab: behavioral interactions with a native crab species, the blue crab. Biological Invasions, 9(7), 837-848. Beguiraj, S.; Kashta, L. 2010. The establishment of blue crab <i>Callinectes sapidus</i> Rathbun, 1896 in the Lagoon of Patok, Albania (south-east Adriatic Sea). Aquatic Invasions 5(2): 219-221. Gollasch S, Macdonald E, Belson S, Botnen H, Christensen JT, Hamer JP, Houvenaghel G, Jelmert A, Lucas I, Masson D, McCollin T, Olenin S, Persson A, Wallentinus I, Wetsteyn LPMJ, Wittling T (2002) Life in Ballast Tanks In: Invasive aquatic species of Europe - distribution, impact and management. Leppäkoski, E., S. Gollasch & S. Olenin (eds). Kluwer Academic Publishers: 217-231.

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Arthropoda	<i>Caprella mutica</i>	East Asia	<i>C. mutica</i> is an aggressive species, out-competing the native European caprellid <i>Caprella linearis</i> for space, even at low densities.	Lowry, J. (2015). <i>Caprella mutica</i> . In: Horton et al. W. World Amphipoda Database /WoRMS; Sergej Olenin/AquaNIS	Ballast water; Recreational boating can also represent an important vector of transport.	Cook EJ, Jahnke M, Kerckhof F, Minchin D, Faasse M, Boos K, Ashton G (2007) European expansion of the introduced amphipod <i>Caprella mutica</i> Schurin 1935. <i>Aq Inv</i> 2: 411-421.
Arthropoda	<i>Chelicorophium curvispinum</i>	Rivers that flow into the Caspian and Black Sea	Ecosystem impact: one of the most successful invaders of European fresh and brackish waters. Rapid growth rate, early maturation and high fecundity allowed this species to form extremely high densities in a number of invaded waterbodies.	Aleksas Narščius/AquaNIS ; Lowry, J. (2015). <i>Chelicorophium curvispinum</i> (G.O. Sars, 1895). In: Horton et al. W. World Amphipoda Database /WoRMS	As the Caspian mud shrimp can also attach to ship hulls, it is likely that the species spread to West and North Europe via inland shipping.	Haas G, Brunke M, Strei B (2002) Fast turnover in dominance of exotic species in the Rhine River determines biodiversity and ecosystem function: an affair between amphipods and mussels. In: Invasive Aquatic Species of Europe: Distribution, Impacts and Management [ed. by Leppakoski E, Gollasch S, Olenin] Dordrecht, The Netherlands: Kluwer Academic Publishers, 426-432. Grabowski M, Jazdzewski K, Konopacka A (2007) Alien Crustacea in Polish waters - Amphipoda. <i>Aquatic Invasions</i> , 2:25-38
Arthropoda	<i>Dikerogammarus villosus</i>	Ponto-Caspian region	Ecosystem impact: <i>D. villosus</i> significantly changes natural food webs of invaded ecosystems and occupies high trophic levels comparable to fish. It is called the killer shrimp.	Sergej Olenin/AquaNIS; Lowry, J. <i>Dikerogammarus villosus</i> . In: Horton et al. W. World Amphipoda Database /WoRMS	Ships; Natural dispersion	Kelleher B, Velde Gder, Giller PS, Bij Vaate Ade, (1999) Dominant role of exotic mass invaders in the diet of important fish species of the River Lower Rhine, The Netherlands. <i>Crustacean Issues</i> , 12:35-46 Dick JTA, Platvoet D (2000) Invading predatory crustacean <i>Dikerogammarus villosus</i> eliminates both native and exotic species. <i>Proceedings of the Royal Society of London. Series B, Biological Sciences</i> , 267(1447):977-983. MacNeil C, Platvoet D (2005) The predatory impact of the freshwater invader <i>Dikerogammarus villosus</i> on native <i>Gammarus pulex</i> (Crustacea: Amphipoda); influences of differential microdistribution and food resources. <i>Journal of Zoology</i> , 267:31-38.

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Arthropoda	<i>Eriocheir sinensis</i>	China	Competes for space and food especially during mass developments. It has an opportunistic diet that includes algae, detritus, and a variety of benthic macroinvertebrates	Anastasija Zaiko/AquaNIS; Davie, P. (2015). <i>Eriocheir sinensis</i> H. Milne Edwards, 1853/WoRMS	ballast water	Rudnick D, Veldhuizen T, Tullis R, Culver C, Hieb K, Tsukimura B (2005) A life history model for the San Francisco Estuary population of the Chinese mitten crab, <i>Eriocheir sinensis</i> (Decapoda: Grapsoidea). Biological Invasions, Volume 7:2, 333 – 350. Gollasch S, Macdonald E, Belson S, Botnen H, Christensen JT, Hamer JP, Houvenaghel G, Jelmert A, Lucas I, Masson D, McCollin T, Olenin S, Persson A, Wallentinus I, Wetsteyn LPMJ, Wittling T (2002) Life in Ballast Tanks In: Invasive aquatic species of Europe - distribution, impact and management. Leppäkoski, E., S. Gollasch & S. Olenin (eds). Kluwer Academic Publishers: 217-231
Arthropoda	<i>Gammarus tigrinus</i>	North America	Ecosystem impact: modifications on native communities; substitution of autochthonous species by competition	Anna Occhipinti / AquaNIS	Ballast water	Jazdzewski, K., Konopacka, A., & Grabowski, M. (2004). Recent drastic changes in the gammarid fauna (Crustacea, Amphipoda) of the Vistula River deltaic system in Poland caused by alien invaders. Diversity and Distributions, 10(2), 81-87.
Arthropoda	<i>Hemigrapsus sanguineus</i>	Northwest Pacific	When occurring in high densities, this species can play an important role in restructuring the prey communities in intertidal habitats and have the potential to affect populations of native species by disrupting the food web.	Davie, P. (2015). <i>Hemigrapsus sanguineus</i> (De Haan, 1835)/WoRMS; Anastasija Zaiko/AquaNIS	Ballast water; Hull fouling	McDermott, J.J. 1998. The western Pacific brachyuran ( <i>Hemigrapsus sanguineus</i> : Grapsidae), in its new habitat along the Atlantic coast of the United States: geographic distribution and ecology. – ICES Journal of Marine Science, 55: 289–298. Brousseau DJ, Baglivo JA (2005) Laboratory Investigations of Food Selection by the Asian Shore Crab, <i>Hemigrapsus sanguineus</i> : Algal versus Animal Preference. Journal of Crustacean Biology 25: 130-134. McDermott J (1998) The western Pacific brachyuran <i>Hemigrapsus sanguineus</i> (Grapsidae) in its new habitat along the Atlantic coast of the United States: reproduction. Journal of Crustacean Biology 18: 308-316 McDermott, J.J. 1998. The western Pacific brachyuran ( <i>Hemigrapsus sanguineus</i> : Grapsidae), in its new habitat along the Atlantic coast of the United States: geographic distribution and ecology. – ICES Journal of Marine Science, 55: 289–298. Jean-Claude Dauvin JC, Tous RiusA, Ruellet T (2009) Recent expansion of two invasive crabs species <i>Hemigrapsus sanguineus</i> (de Haan, 1835) and <i>H. takanoi</i> Asakura and Watanabe 2005 along the Opal Coast, France. Aquatic Invasions 4: 451-465.



Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Arthropoda	<i>Hemigrapsus takanoi</i>	Northwest Pacific	Possibly outcompetes with the native <i>Carcinus maenas</i> , because it has similar habitat preferences.	Ng, P. (2015). <i>Hemigrapsus takanoi</i> Asakura & Watanabe, 2005. /WoRMS; Anna Occhipinti / AquaNIS	Ballast water; Hull fouling	Boets, P., Lock, K., & Goethals, P. L. (2012). Assessing the importance of alien macro-Crustacea (Malacostraca) within macroinvertebrate assemblages in Belgian coastal harbours. <i>Helgoland Marine Research</i> , 66(2), 175-187. Gollasch S (1999) The Asian decapod <i>Hemigrapsus penicillatus</i> (de Haan, 1835) (Grapsidae, Decapoda) introduced in European waters: status quo and future perspective. <i>Helgoländer Meeresuntersuchungen</i> 52: 359-366.
Arthropoda	<i>Limnomysis benedeni</i>	Black, Mediterranean and Caspian Seas	Modification of ecosystem performance and/or addition of new, or reduction of existing, functional groups; Among possible host species of burn spot disease in aquaculture.	CABI. Invasive Species Compendium. Wallingford, UK: CABI International <a href="http://www.cabi.org/isc">www.cabi.org/isc</a> .	Intentional; Aquarium trade; Interconnected waterways; Ballast water	Olenin S, Minchin D, Daunys D, 2007. Assessment of biopollution in aquatic ecosystems. <i>Marine Pollution Bulletin</i> , 55(7-9):379-394. Grigorovich IA, MacIsaac HJ, Shadrin NV, Mills EL, 2002. Patterns and mechanisms of aquatic invertebrate introductions in the Ponto-Caspian region. <i>Canadian Journal of Fisheries and aquatic Sciences</i> , 59:1189-1208. Austin B, Alderman DJ, 1987. Bacterial shell disease of crustaceans. Identification leaflets for diseases and parasites of fish and shellfish., 1-4. Ricciardi A, Rasmussen JB, 1998. Predicting the identity and impact of future biological invaders: a priority for aquatic resource management. <i>Canadian Journal of Fisheries and aquatic Sciences</i> , 55:1759-1765. Wittmann KJ, Ariani AP, 2009. Reappraisal and range extension of non-indigenous Mysidae (Crustacea, Mysida) in continental and coastal waters of eastern France. <i>Biological Invasions</i> , 11(2):401-407.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Arthropoda	<i>Platorchestia platensis</i>	Areas in the NW Atlantic Ocean; Originally described to Uruguay. Unknown	Competition with native species has been demonstrated for Swedish populations; <i>P. platensis</i> has been used as a biomonitor for heavy metals both in Hong Kong and in Denmark. Trials have been made to produce a feed for ornamental fish based on this species.	Van Guelpen, L., G. Pohle, E. Vanden Berghe, and M.J. Costello /NWARMS; WoRMS; Kathe R. Jensen/NOBANIS	Shipping	Jensen, Kathe R. (2010): NOBANIS – Invasive Alien Species Fact Sheet – <i>Platorchestia platensis</i> – From: Identification key to marine invasive species in Nordic waters – NOBANIS <a href="http://www.nobanis.org">www.nobanis.org</a> Katsanevakis, S.; Bogucarskis, K.; Gatto, F.; Vandekerckhove, J.; Deriu, I.; Cardoso A.S. (2012). Building the European Alien Species Information Network (EASIN): a novel approach for the exploration of distributed alien species data. <i>BioInvasions Records</i> . 1: 235-245. Dahl, E. 1946. The Amphipoda of the Sound. Part 1, Terrestrial Amphipoda. <i>Undersökningar över Öresund</i> 29. Lunds Universitets Årsskrift N.F. 2, 42: 1-53. Karlbrink, F. 1969. Distribution and dispersal of Talitridae (Amphipoda) in southern Sweden. <i>Oikos</i> 20: 327-334. Casini, S. and Depledge, M.H. 1997. Influence of copper, zinc, and iron on cadmium accumulation in the talitrid amphipod, <i>Platorchestia platensis</i> . <i>Bulletin of Environmental Contamination and Toxicology</i> 59: 500-506. Rainbow, P.S. 1992. The talitrid amphipod <i>Platorchestia platensis</i> as a potential biomonitor of copper and zinc in Hong Kong: laboratory and field studies. In: <i>The Marine Flora and Fauna of Hong Kong and Southern China IV</i> (ed. B. Morton), pp. 599-610. University of Hong Kong Press, Hong Kong.
Arthropoda	<i>Rhithropanopeus harrisi</i>	NW Atlantic	Although it is a successful invader in many parts of the world, its impacts have not been studied throughout much of its introduced range.	Fofonoff, P.W.; Ruiz, G.M.; Steves, B.; Carlton, J.T. (2014)/NEMESIS; WoRMS	Natural dispersion, ships' ballast water	Fofonoff, P.W.; Ruiz, G.M.; Steves, B.; Carlton, J.T. (2014). National Exotic Marine and Estuarine Species Information System (NEMESIS).

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<b>Mollusca</b>	<i>Magallana gigas</i>	NW Pacific	Competes for food and space with native mussels. Positive impact: creates highly-valued oyster reefs (Troost, 2010 apud Katsanevakis et al., 2014).	Dan Minchin and Stephan Gollasch/DAISIE	Deliberate introduction; Also recorded attached to ships' hulls and in ballast water.	Diederich S (2006) High survival and growth rates of introduced Pacific oysters may cause restrictions on habitat use by native mussels in the Wadden Sea. <i>Journal of Experimental Marine Biology and Ecology</i> 328(2):211-227 Heral M (1989) Traditional oyster culture in France. Pages 342-387 in G. Barnabé, J.F. Solbé and L. Laird, editors. <i>Aquaculture Volume 1</i> . Ellis Horwood London, pp 342-387 Wolff WJ, Reise K (2002) Oyster imports as a vector for the introduction of alien species into northern and western European coastal waters. In: Leppäkoski E, Gollasch S, Olenin S (eds) <i>Invasive aquatic species of Europe: Distribution, Impact and Management</i> Kluwer, Dordrecht, the Netherlands, pp 193-205
<b>Mollusca</b>	<i>Crepidula fornicata</i>	North America	Often associated with oyster layings and on scallop beds; It also fouls artificial structures in port regions. It competes with native commercial bivalves.	Dan Minchin/DAISIE	Aquaculture, hull fouling, floating structures and natural dispersal.	Blanchard M (1996) Spread of the slipper limpet <i>Crepidula fornicata</i> (L., 1758) in Europe. Current state and consequences. <i>Scientia Marina</i> 61(Suppl. 2): 109-118 Minchin D, McGrath D, Duggan CB (1995) The slipper limpet, <i>Crepidula fornicata</i> (L.), in Irish waters, with a review of its occurrence in the north-eastern Atlantic. <i>J Conch Lond</i> , 35: 247-254 Walne PR (1956) The biology and distribution of the slipper limpet <i>Crepidula fornicata</i> in Essex rivers with notes on the distribution of larger epi-benthic invertebrates. <i>Fish Invest Lond</i> (2) 20: No 6: 1-52
<b>Mollusca</b>	<i>Dreissena polymorpha</i>	Black Sea	The high reproductive output and ability to extend their planktonic stage enables <i>D. polymorpha</i> to disperse rapidly. Outcompetes native species for resources and/or space; Causes damage to marine structures or archaeology.	Romualda Chuševè, Anastasija Zaiko/AquaNIS ; Rosenberg, G.; Rosenberg, G.; Huber, M. 2015. <i>Dreissena polymorpha</i> (Pallas, 1771). In: <i>MolluscaBase</i> (2015)/WoRMS.	Ballast water; biofouling.	Gollasch, S. and Leppäkoski, E (eds.). 1999. Initial Risk Assessment of Alien Species in Nordic Coastal Waters. Nordic Council of Ministers, Copenhagen. Gollasch S, Macdonald E, Belson S, Botnen H, Christensen JT, Hamer JP, Houvenaghel G, Jelmert A, Lucas I, Masson D, McCollin T, Olenin S, Persson A, Wallentinus I, Wetsteyn LPMJ, Wittling T (2002) Life in Ballast Tanks In: <i>Invasive aquatic species of Europe - distribution, impact and management</i> . Leppäkoski, E, Gollasch S, Olenin S(eds). Kluwer Academic Publishers: 217-231. Lalaguna, C.D.; Marco, A.A. (2008). The zebra mussel invasion in Spain and navigation rules. <i>Aquatic Invasions</i> 3(3): 315-324.

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Mollusca	<i>Ensis directus</i>	USA	Outcompetes native species for resources and/or space; Local economy can benefit from this species, as it can be fished and consumed; the species may serve as food for sea birds, but it might negatively influence biodiversity.	VLIZ Alien Species Consortium; von Cosel, R.; Gofas, S. (2015). <i>Ensis directus</i> (Conrad, 1843). In: MolluscaBase (2015)/WoRMS	Natural drift	Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. <i>Aquatic Invasions</i> . 8(1): 3-19.
Mollusca	<i>Mya arenaria</i>	NW Atlantic	Observed impacts on fisheries and aquaculture; Described impacts on biodiversity.	Gofas, S. 2015. <i>Mya arenaria</i> Linnaeus, 1758. In: MolluscaBase (2015). /WoRMS ; Anastasija Zaiko/AquaNIS	Ballast water; Hull fouling; Oyster stocking	Gollasch S, Macdonald E, Belson S, Botnen H, Christensen JT, Hamer JP, Houvenaghel G, Jelmert A, Lucas I, Masson D, McCollin T, Olenin S, Persson A, Wallentinus I, Wetsteyn LPMJ, Wittling T (2002) Life in Ballast Tanks In: <i>Invasive aquatic species of Europe - distribution, impact and management</i> . Leppäkoski, E, Gollasch S, Olenin S(eds). Kluwer Academic Publishers: 217-231. Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i> . 6(9): 485-492. Katsanevakis S, Wallentinus I, Zenetos A, Leppakoski E, Cinar ME, Ozturk B, Grabowski M, Golani D, Cardoso AC. 2014. Impacts of invasive alien marine species on ecosystem services and biodiversity: a pan-European review. <i>Aquatic Invasions</i> 9: 391-423.
Mollusca	<i>Petricolaria pholadiformis</i>	East coast of North America	This bivalve drills passageways in peat blocks, wood, hard clay and limestone.	Huber, M.; Gofas, S. 2015. <i>Petricolaria pholadiformis</i> (Lamarck, 1818). In: MolluscaBase (2015)/WoRMS	It was brought to Europe (England) around 1890 together with the transport of the eastern oyster <i>Crassostrea virginica</i> .	Gofas, S.; Le Renard, J.; Bouchet, P. (2001). Mollusca. in: Costello, M.J. et al. (Ed.) (2001). <i>European register of marine species: a checklist of the marine species in Europe and a bibliography of guides to their identification</i> . Collection Patrimoines Naturels. 50: pp. 180-213. Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i> . 6(9): 485-492.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Mollusca	<i>Potamopyrgus antipodarum</i>	New Zealand	Outcompetes with native species for resources and/or space; These snails prey on young oysters	Marshall, B.; Gofas, S. 2015. <i>Potamopyrgus antipodarum</i> . In: MolluscaBase (2015)/WoRMS	Ships; Natural dispersion	Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. Aquatic Invasions. 8(1): 3-19. Ramos, M. (2010). IBERFAUNA. The Iberian Fauna Databank. available online at <a href="http://iberfauna.mncn.csic.es/">http://iberfauna.mncn.csic.es/</a>
Mollusca	<i>Rapana venosa</i>	Sea of Japan, Yellow Sea, Bohai Sea, and the East China Sea to Taiwan	Represents risk in areas with oyster cultures (prey on oysters); In the North Sea the whelk may represent a competitor of the native whelk <i>Buccinum undatum</i>	Stephan Gollasch/DAISIE; Houart, R.; Gofas, S. (2015). <i>Rapana venosa</i> (Valenciennes, 1846). In: MolluscaBase (2015)/WoRMS	Most likely vector is oyster shipments; Ballast water and hull fouling (possibilities)	Kerckhof F, Vink RJ, Nieweg DC, Post JN (2006) The veined whelk <i>Rapana venosa</i> has reached the North Sea. Aquatic Invasions 1:35-37 Mann R, Occhipinti A, Harding JM (eds) (2004) Alien Species Alert: <i>Rapana venosa</i> (veined whelk). ICES Cooperative Research Report No. 264. [available at <a href="http://www.ices.dk">www.ices.dk</a> ] Zolotarev V (1996) The Black Sea ecosystem changes related to the introduction of new mollusc species. P.S.Z.N. I: Marine Ecology 17(1–3):227–236
Mollusca	<i>Urosalpinx cinerea</i>	NW Atlantic	Loss of aquaculture/commercial/recreational harvest or gain; Prey on oysters	Houart, R.; Gofas, S. 2015. <i>Urosalpinx cinerea</i> (Say, 1822). In: MolluscaBase (2015)/WoRMS; NWARMS	Fisheries: accidental with deliberate translocations of fish or shellfish	Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. Aquatic Invasions. 8(1): 3-19. Faasse, M.; Ligthart, M. (2009). American ( <i>Urosalpinx cinerea</i> ) and Japanese oyster drill ( <i>Ocenebrellus inornatus</i> ) (Gastropoda: Muricidae) flourish near shellfish culture plots in The Netherlands. Aquatic Invasions 4(2): 321-326.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Mollusca</b>	<i>Venerupis philippinarum</i>	Japan, Korea and China	Observed impacts on fisheries and aquaculture and on biodiversity.	Gofas, S.; Huber, M. 2015. <i>Venerupis philippinarum</i> (A. Adams & Reeve, 1850). In: MolluscaBase (2015)/WoRMS	Fisheries: deliberate translocations of fish or shellfish to establish or support fishery; Shipping	Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. <i>Aquatic Invasions</i> . 8(1): 3-19. Katsanevakis, S.; Bogucarskis, K.; Gatto, F.; Vandekerckhove, J.; Deriu, I.; Cardoso A.S. (2012). Building the European Alien Species Information Network (EASIN): a novel approach for the exploration of distributed alien species data. <i>BioInvasions Records</i> . 1: 235-245. Cohen, Andrew N. 2011. The Exotics Guide: Non-native Marine Species of the North American Pacific Coast. Center for Research on Aquatic Bioinvasions, Richmond, CA, and San Francisco Estuary Institute, Oakland, CA. Revised September 2011. Katsanevakis S, Wallentinus I, Zenetos A, Leppakoski E, Cinar ME, Ozturk B, Grabowski M, Golani D, Cardoso AC. 2014. Impacts of invasive alien marine species on ecosystem services and biodiversity: a pan-European review. <i>Aquatic Invasions</i> 9: 391-423.
<b>Ascidacea</b>	<i>Styela clava</i>	The Sea of Othotsk, Korea and Siberia	Its high biomass results in competition with other filter-feeders; Sprays produced from damaged tissues when removing them from oysters are known to result in a respiratory condition in humans; It can foul artificial structures in port regions, oysters and shellfish held in hanging culture; It may also impede fishing activities.	Dan Minchin/DAISIE	Probably introduced to Europe as fouling on warships arriving during the Korean War; Vessels hulls. Possible spread with oyster stock; Ships' ballast water; Floating port structures.	Davis MH, Lützen J, Davis ME. (2007) The spread of <i>Styela clava</i> , Herdman 1882 (Tunicata: Ascidaceae) in European waters. Lützen J (1999) <i>Styela clava</i> Herdman (Urochordata, Ascidaceae) a successful immigrant to northwest Europe: ecology, propagation and chronology of spread. <i>Helgoländer Meeresunters</i> 52:383-391 Parker LE, Culloty S, O' Riordan RM, Kelleher B, Steele S, and van der Velde G (1999) Preliminary study on the gonad development of the exotic ascidian <i>Styela clava</i> in Cork Harbour, Ireland. <i>Journal of the Marine Biological Association of the United Kingdom</i> 79:1141-1142

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Bryozoa	<i>Tricellaria inopinata</i>	Cryptogenic in temperate Pacific: North America, Japan to Taiwan, Australia	Its presence is associated to a reduction in frequency and abundance of native bryozoan species. It is a fast growing fouling organism, settling on buoys, vessels and ropes.	B. S. Galil and A. Occhipinti-Ambrogi/DAISIE	Oyster import; Secondary introductions are possibly due to small vessels or dispersed with currents on floating fragments of Sargassum.	Blauwe H de, Faasse M (2001) Extension of the range of the bryozoans <i>Tricellaria inopinata</i> and <i>Bugula simplex</i> in the north-east Atlantic Ocean (Bryozoa: Cheilostomatida). Nederlandse Faunistische Mededelingen 14:103-112 Dyrynda PEJ, Fairall VR, Occhipinti Ambrogi A, d'Hondt JL (2000) The distribution, origin and taxonomy of <i>Tricellaria inopinata</i> d'Hondt and Occhipinti Ambrogi, 1985, and invasive bryozoan new to the Atlantic. Journal of Natural History 34:1993-2006 Hondt JL d', Occhipinti Ambrogi A (1985) <i>Tricellaria inopinata</i> , n. sp., un nouveau Bryozaire Cheilostome de la faune méditerranéenne. P.S.Z.N.I: Marine Ecology 6(1):35-46
Cnidaria	<i>Gonionemus vertens</i>	NW Pacific	Stings from <i>G. vertens</i> are unusually venomous, and it can be dangerous to swimmers.	Anna Occhipinti (2012)/iAquaNIS; Schuchert, P. (2016)/WoRMS	Fisheries: accidental with deliberate translocations of fish or shellfish	Tambs-Lyche H, (1964). <i>Gonionemus vertens</i> L. Agassiz (Limnomedusae) a Zoogeographical puzzle. Sarsia. 15:1-8 Minchin, D.; Cook, E.; Clark, P. (2013). Alien species in British brackish and marine waters. Aquatic Invasions. 8(1): 3-19.

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Ctenophora</b>	<i>Mnemiopsis leidyi</i>	NW, SW Atlantic	<i>Mnemiopsis leidyi</i> is a major zooplankton predator and is associated with fishery crashes (Costello, 2001). A cascading effect occurred at the higher trophic levels, from a decrease in zooplankton stock and collapsing planktivorous fish, to vanishing predatory fish and dolphins. Similar effects occurred at lower trophic levels: from a decrease in zooplankton stock to an increase in phytoplankton, which was released from zooplankton grazing pressure. The majority of these effects were top-down, but a few were also bottom-up.	Costello J, Mianzan H, Shiganova T (2005) <i>Mnemiopsis leidyi</i> (comb jelly) In: Global Invasive Species Database.	Ballast water	Purcell JE, Shiganova TA, Decker MB, Houde ED (2001) The ctenophore <i>Mnemiopsis</i> in native and exotic habitats: U.S. estuaries versus the Black Sea basin. <i>Hydrobiologia</i> 451: 145-176 Reusch et al 2010. Microsatellites reveal origin and genetic diversity of Eurasian invasions by one of the world's most notorious marine invader, <i>Mnemiopsis leidyi</i> (Ctenophora). <i>Molecular Ecology</i>
<b>Perciformes</b>	<i>Carassius gibelio</i>	Usually considered as native from central Europe to Siberia or introduced to European waters from eastern Asia.	May pose a threat to humans; Host to parasitic, endoparasitic species. In aquaculture systems, <i>C. gibelio</i> is an unwelcome competitor with cultures of the major reared species.	Bailly, N. 2015. <i>Carassius gibelio</i> (Bloch, 1782). In: Froese, R. and D. Pauly. Editors. (2015) FishBase/WoR MS; CABI International <a href="http://www.cabi.org/isc">www.cabi.org/isc</a> .	<i>C. gibelio</i> was intentionally introduced either to Belarus or Poland for stock enhancement, recreational and aquaculture purposes, with a secondary dispersal route being the central corridor	Kottelat, M. and J. Freyhof, 2007. Handbook of European freshwater fishes. Publications Kottelat, Cornol and Freyhof, Berlin. 646 pp. Semenchenko V, Grabowska J, Grabowski M, Rizevsky V, Pluta M, 2011. Non-native fish in Belarusian and Polish areas of the European central invasion corridor. <i>Oceanological and Hydrobiological Studies</i> , 40(1):57- 67.



Group	Species	Native range	known impacts	Source	Vectors / pathways	References
Perciformes	<i>Neogobius melanostomus</i>	Black Sea	Outcompetes with native fish for food.	Bailly, N. 2015. <i>Neogobius melanostomus</i> (Pallas, 1814). In: Froese, R. and D. Pauly. Editors. (2015) FishBase./WoRMS; Henn Ojaveer/AquaNIS	Canals: natural range expansion through man-made canals; Ballast water	Ricciardi, A., & Rasmussen, J. B. (1998). Predicting the identity and impact of future biological invaders: a priority for aquatic resource management. <i>Canadian Journal of Fisheries and Aquatic Sciences</i> , 55(7), 1759-1765.
Perciformes	<i>Oncorhynchus mykiss</i>	USA; Native to the Pacific coastal inland waters.	Impacts include hybridisation, disease transmission, predation and competition with native species. Consumes native species.	Bailly, N. 2015. <i>Oncorhynchus mykiss</i> (Walbaum, 1792). In: Froese, R. and D. Pauly. Editors. (2015) FishBase /WoRMS; NWARMS; <i>Oncorhynchus mykiss</i> . Global Invasive Species Database, 2005.	Fisheries; Aquaculture	Streftaris, N.; Zenetos, A.; Papathanassiou, E. (2005). Globalisation in marine ecosystems: the story of non-indigenous marine species across European seas. Hayes, K.; Sliwa, C.; Migus, S.; McEnnulty, F.; Dunstan, P.; Heritagearkes, P. (2005). National priority pests. Part II, Ranking of Australian marine pests. Australian Government Department of the Environment and Heritage: Parkes. ISBN 1 876996 80 3. 94 pp.Oceanogr. Mar. Biol. Ann. Rev. 43: 419-453.
Perciformes	<i>Salvelinus fontinalis</i>	East North America	Competes with and predated on native fish such as other salmonids for food and cover; Sport fishing for brook trout has a positive economic effect for local communities.	Melanie Josefsson/DAISIE; Bailly, N. 2015. <i>Salvelinus fontinalis</i> (Mitchill, 1814). In: Froese, R. and D. Pauly. Editors. (2015) FishBase; Global Invasive Species Database (2017) Species profile: <i>Salvelinus fontinalis</i> .	Intentionally introduced for aquaculture for sport fisheries and for food production.	FishBase (2006) <i>Salvelinus fontinalis</i> , Brook trout. FishBase World Wide Web electronic publication. URL: <a href="http://www.fishbase.org/Summary/speciesSummary.php?ID=246&amp;genusname=Salvelinus&amp;speciesname=fontinalis">www.fishbase.org/Summary/speciesSummary.php?ID=246&amp;genusname=Salvelinus&amp;speciesname=fontinalis</a> . Jansson K (2006) Nobanis Invasive Aliens Fact Sheet – <i>Salvelinus fontinalis</i> . From online database of the North European and Baltic Network on Invasive Alien Species. NOBANIS <a href="http://www.nobanis.org">www.nobanis.org</a> .



## **Annex 2**

**Table 2-3 (chapter 2): Invasive marine and oligohaline species recorded  
for the SW Atlantic Ocean**

**Table 2.3: Invasive marine and oligohaline species recorded for SW Atlantic Ocean.**

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Dinoflagellata</b>	<i>Alexandrium tamarense</i>	Widely distributed coastal and estuarine dinoflagellate species, mainly found in cold to cold-temperate waters in North America, Europe and Japan.	Potential effect on the marine biota due to oxygen depletion and temporary exclusion of other species of phytoplankton during the occurrence of blooms; bloom events of <i>A. tamarense</i> have been linked to several massive fish kills. <i>Alexandrium tamarense</i> is a known toxin-producing dinoflagellate species. This species produces neurotoxins which can affect humans, other mammals, fish and birds.	Fraga, S./WoRMS; MMA, 2009; M.A. Faust & R.A. Gulledge/Harmful Marine Dinoflagellates ( <a href="http://species-identification.org/">http://species-identification.org/</a> )	Water column, tides, and in ships' ballast water.	Steidinger, K.A. and K. Tangen 1996. Dinoflagellates. In: C.R. Tomas (ed), Identifying Marine Diatoms and Dinoflagellates, Academic Press, New York: 387-598. Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i> . 6(9): 485-492. Hallegraeff, G.M.; Anderson, D.M.; Cembella, A.D. Manual on harmful marine microalgae. Monographs on Oceanographic Methodologies 11. Paris: Unesco, 2003. 793 p. Larsen, J. and O. Moestrup 1989. Guide to Toxic and Potentially Toxic Marine Algae. The Fish Inspection Service, Ministry of Fisheries, Copenhagen. 61 pp. Hallegraeff, G.M. 1991. Aquaculturists Guide to Harmful Australian Microalgae. Fishing Industry Training Board of Tasmania/CSIRO Division of Fisheries, Hobart, 111 pp. Proença, L.A.O.; Fernandes, L.F. Introdução de microalgas no ambiente marinho: impactos negativos e fatores controladores. In: SILVA, J.S.V.; SOUZA, R.C.C.L (Org.). Água de lastro e bioinvasão. Rio de Janeiro: Ed. Interciência, 2004. p. 77-97.  MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33).

Group	Species	Native range	known impacts	Source	Vectors / pathways	References
<b>Bacillariophyceae</b>	<i>Coscinodiscus wailesii</i>	North Pacific	Dense blooms associate to fisheries and aquaculture losses	WoRMS	Ballast water; aquaculture	<p>Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i>. 6(9): 485-492.</p> <p>Proença, L. &amp; Fernandes, L. 2004. Introdução de microalgas no ambiente marinho: impactos negativos e fatores controladores. Pages 77-97 in Silva JaS, RCCL, ed. <i>Água de Lastro e Bioinvasão</i>. Rio de Janeiro: Interciencia.</p> <p>KRABERG, A. BAUMANN, M &amp; DÜRSELEN, C-D. 2010. <i>Coastal Phytoplankton - Photo Guide for Northern European Seas</i>. 204 pp. Munchen: Verlag Friedrich Pfeil.</p>
<b>Ochrophyta</b>	<i>Undaria pinnatifida</i>	Northern Asia	Negative impact on algal biodiversity. Positive impact due to the provision of new habitat structures (e.g. higher benthic invertebrate richness and diversity).	Guiry, Michael D. (2015). In: Guiry, M.D. & Guiry, G.M. (2015). <i>AlgaeBase</i> . World-wide electronic publication, National University of Ireland, Galway / WoRMS	Ballast water	<p>Meretta, P.E.; Matula, C.V.; Casas, G. (2012). Occurrence of the alien kelp <i>Undaria pinnatifida</i> (Laminariales, Phaeophyceae) in Mar del Plata, Argentina. <i>BioInvasions Records</i> 1(1): 59–63.</p> <p>Piriz ML, Casas G (1994) Occurrence of <i>Undaria pinnatifida</i> in Golfo Nuevo Argentina. <i>Applied Phycology Forum</i> 10: 4</p> <p>Casas GN, Scrosati R, Piriz ML (2004) The invasive kelp <i>Undaria pinnatifida</i> (Phaeophyceae, Laminariales) reduces native seaweed diversity in Nuevo Gulf (Patagonia, Argentina). <i>Biological Invasions</i> 6: 411-416</p> <p>Irigoyen A, Trobbiani G, Sgarlatta M, Raffo M (2011) Effects of the alien algae <i>Undaria pinnatifida</i> (Phaeophyceae, Laminariales) on the diversity and abundance of benthic macrofauna in Golfo Nuevo (Patagonia, Argentina): potential implications for local food webs. <i>Biological Invasions</i> 13(7): 1521-1532</p> <p>Orensanz, J. M.; Schwindt, E.; Pastorino, G.; Bortolus, A.; Casas, G.; Darrigrán, G.; Elías, R.; López-Gappa, J. J.; Obenat, S.; Pascual, M.; Penchaszadeh, P.; Piriz, M. L.; Scarabino, F.; Spivak, E. D.; Villarino, E. A. (2002). No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. <i>Biological Invasions</i>. 4: 115-143.</p>

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
<b>Chlorophyta</b>	<i>Caulerpa scalpelliformis</i> var. <i>denticulate</i>	Indo-Pacific Ocean	Displacement of native species; May cause impacts to the local benthic community	MMA, 2009; Guiry, Michael D. (2015). In: Guiry, M.D. & Guiry, G.M. (2015). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway / WoRMS	Fouling; aquaria	Falcão, C.; Széchy, M.T.M. Changes in shallow phytobenthic assemblages in southeastern Brazil, following the replacement of <i>Sargassum vulgare</i> (Phaeophyta) by <i>Caulerpa scalpelliformis</i> (Chlorophyta). Botanica Marina, v. 48, p. 208-217, 2005.  MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33)
<b>Tracheophyta</b>	<i>Spartina townsendii</i> var. <i>Anglica</i>	Southern England	Ecological impacts of <i>S. anglica</i> are described in general as follows: loss of valuable habitat for endobenthic invertebrates and for migrating shorebirds and waterfowl; loss of rearing habitat for fish; replacement of native plants and more diverse native plant communities; and alteration the course of succession. <i>Spartina anglica</i> has the potential to be used for economic benefits and has been used as an agent for coastal protection and stabilization, which can also cause changes in water circulation patterns.	NOBANIS ( <a href="https://www.nobanis.org">https://www.nobanis.org</a> ); WoRMS (2014). <i>Spartina townsendii</i> var. <i>anglica</i> C.E. Hubbard. In: Costello, M.J.; Bouchet, P.; Boxshall, G.; Arvantidis, C.; Appeltans, W. (2014) European Register of Marine Species	Ballast water; deliberate introduction	Nehring, S. and Adersen, H. (2006): NOBANIS – Invasive Alien Species Fact Sheet – <i>Spartina anglica</i> . – From: Online Database of the European Network on Invasive Alien Species - NOBANIS ( <a href="http://www.nobanis.org">www.nobanis.org</a> ). Orensanz, J. M.; Schwindt, E.; Pastorino, G.; Bortolus, A.; Casas, G.; Darrigrán, G.; Elías, R.; López-Gappa, J. J.; Obenat, S.; Pascual, M.; Penchaszadeh, P.; Piriz, M. L.; Scarabino, F.; Spivak, E. D.; Villarino, E. A. (2002). No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. Biological Invasions. 4: 115-143.

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
<b>Annelida</b>	<i>Ficopomatus enigmaticus</i>	Unknown	Status of invasiveness varies from invasive to uncertain; Some associated impacts are loss of aquaculture/commercial/recreational harvest or gain and adverse habitat modification in Uruguay.	Read, G.; ten Hove, H. (2015). <i>Ficopomatus enigmaticus</i> . In: Read, G.; Fauchald, K. (Ed.) (2015) World Polychaeta database / WoRMS	Ballast water, hull fouling	Fofonoff, P.W.; Ruiz, G.M.; Steves, B.; Carlton, J.T. 2003. National Exotic Marine and Estuarine Species Information System (NEMESIS). Rioja, E. (1943) Estudios Anelidológicos IX: La presencia de la <i>Mercierella enigmatica</i> Fauvel, en las costas argentinas, Anales del Instituto de Biología de la Universidad Nacional Autónoma de México 14: 547-551. Orensanz, J. M.; Schwindt, E.; Pastorino, G.; Bortolus, A.; Casas, G.; Darrigrán, G.; Elías, R.; López-Gappa, J. J.; Obenat, S.; Pascual, M.; Penchaszadeh, P.; Piriz, M. L.; Scarabino, F.; Spivak, E. D.; Villarino, E. A. (2002). No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. Biological Invasions. 4: 115-143.
<b>Arthropoda</b>	<i>Charybdis hellerii</i>	Indo-Pacific Ocean	It is a potential predator and competitor of native crabs, but its impacts are not well-documented.	Davie, P.; Fransen, C./WoRMS; IUCN's ISSG (Invasive Species Specialist Group). Global Invasive Species Database (GISD); National Exotic Marine and Estuarine Species Information System. <a href="http://invasions.si.edu/nemesis/">http://invasions.si.edu/nemesis/</a> .	Ships: accidental with ballast water, sea water systems, live wells or other deck basins	Fofonoff, P.W.; Ruiz, G.M.; Steves, B.; Carlton, J.T. 2003. National Exotic Marine and Estuarine Species Information System (NEMESIS) Mantelatto, F. L. M.; Diaz, L. L. (1999). Extension of the known distribution of <i>Charybdis helleri</i> (A. Milne Edwards 1967) (Decapoda, Portunidae) along the western tropical South Atlantic. Crustaceana. 72, 617-619. Sant'Anna, B.S.; Watanabe, T.T.; Turra, A.; Zara, F.J. (2012). Relative abundance and population biology of the non-indigenous crab <i>Charybdis hellerii</i> (Crustacea: Brachyura: Portunidae) in a southwestern Atlantic estuary-bay complex. Aquatic Invasions 7(3): 347–356.

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
<b>Arthropoda</b>	<i>Eurytemora americana</i>	NW Pacific	Competes with native species for resources; Caused a decrease on the native population of a native copepod in Bahía Blanca, Argentina.	The Nature Conservancy ( <a href="http://www.nature.org/">http://www.nature.org/</a> )	Ballast water	Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i> . 6(9): 485-492. Guinder VA, Popovich CA, Molinero JC, Marcovecchio J. 2013. Phytoplankton summer bloom dynamics in the Bahía Blanca Estuary in relation to changing environmental conditions. <i>Continental Shelf Research</i> 52: 150-158.
<b>Mollusca</b>	<i>Magallana gigas</i>	NW Pacific	<i>M. gigas</i> , has been delibered introduced in at least 45 ecoregions; Despite the economic gains, the ecological impact of introductions of this species are related to the alteration of ecosystem processes; modifying the abundance or distribution of native species of mussels.	The Nature Conservancy ( <a href="http://www.nature.org/">http://www.nature.org/</a> )	Deliberate introduction; Also recorded attached to ships' hulls and in ballast water.	Orensanz, J. M.; Schwindt, E.; Pastorino, G.; Bortolus, A.; Casas, G.; Darrigrán, G.; Elías, R.; López-Gappa, J. J.; Obenat, S.; Pascual, M.; Penchaszadeh, P.; Piriz, M. L.; Scarabino, F.; Spivak, E. D.; Villarino, E. A. (2002). No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. <i>Biological Invasions</i> . 4: 115-143. Heral M (1989) Traditional oyster culture in France. Pages 342-387 in G. Barnabé, J.F. Solbé and L. Laird, editors. <i>Aquaculture Volume 1</i> . Ellis Horwood London, pp 342-387 A global review of marine invasive species: where they are, how they are being introduced, and which ones are most harmful. Jennifer L Molnar, Rebecca L Gamboa, Carmen Revenga, Mark D Spalding. 2008. Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i> . Vol. 6, No. 9, pp. 485-492.



Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
Mollusca	<i>Isognomon bicolor</i>	Caribbean	Possible competition with local bivalves for space	Huber, M. /WoRMS; MMA,2009	Ballast water	<p>Migotto, A.E.; Tiago, C.G.; Magalhães, A.R.M. Malacofauna marinha da região costeira do canal de São Sebastião, SP, Brasil: Gastropoda, Bivalvia, Polyplacophora e Scaphopoda. Boletim do Instituto Oceanográfico, v. 41, n. 1/2, p. 13-27, 1993.</p> <p>Fernandes, F.C.; Rapagnã, L.C.; Bueno, G.B.D. Estudo da população do bivalve exótico <i>Isognomon bicolor</i> (C.B. Adams, 1845) (Bivalvia, Isognomonidae) na Ponta da Fortaleza em Arraial do Cabo. In: SILVA, J.S.V.; 346 Informe sobre as Espécies Exóticas Invasoras Marinhas no Brasil SOUZA, R.C.C.L (Org.). Água de lastro e bioinvasão. Rio de Janeiro: Ed. Interciência, 2004. p. 133-141.</p> <p>Domaneschi, O.; Martins, C.M. <i>Isognomon bicolor</i> (C.B. Adams) (Bivalvia, Isognomonidae): primeiro registro para o Brasil, redescritção da espécie e considerações sobre a ocorrência e distribuição de <i>Isognomon</i> na costa brasileira. Revista Brasileira de Zoologia, v. 19, n. 2, p. 611-627, 2002.</p> <p>MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33)</p>

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
<b>Mollusca</b>	<i>Rapana venosa</i>	Sea of Japan, Yellow Sea, Bohai Sea, and the East China Sea to Taiwan / W Pacific	Its main diet consists of other mollusk species, representing a risk to oyster cultures. Have caused significant changes in the ecology of bottom-dwelling organisms.	Stephan Gollasch/DAISIE	Most likely vector is oyster shipments; Ballast water and hull fouling (possibilities)	Kerckhof F, Vink RJ, Nieweg DC, Post JN (2006) The veined whelk <i>Rapana venosa</i> has reached the North Sea. Aquatic Invasions 1:35-37 Mann R, Occhipinti A, Harding JM (eds) (2004) Alien Species Alert: <i>Rapana venosa</i> (veined whelk). ICES Cooperative Research Report No. 264. [available at <a href="http://www.ices.dk">www.ices.dk</a> ] Molnar, J. L.; Gamboa, R. L.; Revenga, C.; Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. <i>Frontiers in Ecology and the Environment</i> . 6(9): 485-492. Orensanz, J. M.; Schwindt, E.; Pastorino, G.; Bortolus, A.; Casas, G.; Darrigrán, G.; Elías, R.; López-Gappa, J. J.; Obenat, S.; Pascual, M.; Penchaszadeh, P.; Piriz, M. L.; Scarabino, F.; Spivak, E. D.; Villarino, E. A. (2002). No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. <i>Biological Invasions</i> . 4: 115-143.

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
Mollusca	<i>Limnoperna fortunei</i>	China, SE Asia	The introduction of the golden mussel produced a rapid change in benthic communities and threatened the local biodiversity. Golden mussels settle in high numbers on native bivalves. Its geographic distribution englobes areas located in Argentina, Uruguay, regions south, southeast and central-west from Brazil. A alert bulletin from the CBEI (Centro de Bioengenharia de Espécies Invasoras) from 10th November, 2015 detected this species in the São Francisco basin. The freshwater mussel was reported in estuarine water of up to 3 ppt (Orensanz et al., 2002).	Huber, M. (2015). <i>Limnoperna fortunei</i> (Dunker, 1857). In: MolluscaBase (2015)/WoRMS; IUCN's ISSG (Invasive Species Specialist Group). Global Invasive Species Database (GISD).	Ballast water	Mansur, M. C. D., Santos, C. D., Darrigran, G., Heydrich, I., Callil, C. T., & Cardoso, F. R. 2003. Primeiros dados quali-quantitativos do mexilhão-dourado, <i>Limnoperna fortunei</i> (Dunker), no Delta do Jacuí, no Lago Guaíba e na Laguna dos Patos, Rio Grande do Sul, Brasil e alguns aspectos de sua invasão no novo ambiente. Revista Brasileira de Zoologia, 20(1), 75-84. Darrigran, G., 2000. Invasive Freshwater bivalves of the Neotropical Region. <i>Dreissena</i> , vol. II, no. 2, p. 7-13. França, RS., Suriani, AL. and Rocha, O., 2007. Composição de espécies de moluscos bentônicos nos reservatórios baixo rio Tietê (São Paulo, Brasil) com uma avaliação do impacto causado pelas espécies exóticas invasoras. Rev. Bras. Zool., vol. 24, no. 1, p. 41-51. Pastorino, G., Darrigran, G., Martín, S. and Lunaschi, L., 1993. <i>Limnoperna fortunei</i> (Dunker, 1857) (Mytilidae), Nuevo bivalvo invasor en aguas del Rio de La Plata. Neotropica, vol. 39, no. 101-102, p. 34. Orensanz, J. M.; Schwindt, E.; Pastorino, G.; Bortolus, A.; Casas, G.; Darrigran, G.; Elías, R.; López-Gappa, J. J.; Obenat, S.; Pascual, M.; Penchaszadeh, P.; Piriz, M. L.; Scarabino, F.; Spivak, E. D.; Villarino, E. A. (2002). No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. Biological Invasions. 4: 115-143. Boletim de Alerta nº03, 10/11/2015 - CBEI (Centro de pesquisas e tecnologia em Bioengenharia de Espécies Invasoras: <a href="http://www.cbeih.org">www.cbeih.org</a> ).

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
Mollusca	<i>Myoforceps aristatus</i>	Caribbean	Spiked species can cause great damage to scallop cultivation	MMA,2009	Aquaculture; ballast water; fouling; ocean currents	SIMONE, L.R.L.; GONÇALVES, E.P. Anatomical study on <i>Myoforceps aristatus</i> , an invasive boring bivalve in S.E. Brazilian coast (Mytilidae). Papéis Avulsos de Zoologia, v. 46, p. 57-65, 2006. MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33)
Asciacea	<i>Styela plicata</i>	North Atlantic Ocean; part of Caribbean Sea; Some authors describe the Indo-Pacific region as its putative native range	competes with native organisms for space; Loss of aquaculture, commercial, recreational harvest or gain.	IUCN's ISSG (Invasive Species Specialist Group). Global Invasive Species Database (GISD); Sanamyan, K. /WoRMS; MMA, 2009; Indian River Lagoon Species Inventory /Smithsonian Marine Station at Fort Pierce	Fouling; aquaculture	Fuller, Pam., 2007. <i>Styela plicata</i> . USGS Nonindigenous Aquatic Species Database, Gainesville, FL. Carlton J.T., and M.H. Ruckelshaus. 1997. Nonindigenous marine invertebrates and algae. Pp 187-201 in: Simberloff D., Schmitz D.C., and T.C. Brown (eds). Strangers in Paradise. Island Press, Washington, D.C. 467 p. Lambert C.C., and G. Lambert. 1998. Non-indigenous ascidians in southern California harbors and marinas. Marine Biology 130:675-688. Lambert G. 2001. A global overview of ascidian introductions and their possible impact on the endemic fauna. Pp 249-257 In: Sawada, H., Tokosawa, H., and C.D. Lambert (eds). The Biology of Ascidians. Springer-Verlag, Tokyo, Japan. 470 p. RODRIGUES, S.A.; ROCHA, R.M.M.; LOTUFO, T.M.C. Guia ilustrado para identificação das ascídias do estado de São Paulo. 1. ed. São Paulo: Editora IB/USP/FAPESP, 1998. MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33).

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
Cnidaria	<i>Tubastraea coccinea</i>	Indo-Pacific region	Alters trophic interactions	MMA, 2009; Cairns, S./WoRMS	Oil platform and hull fouling	<p>Nunes FLD, Norris RD, Knowlton N. (2011). Long Distance Dispersal and Connectivity in Amphi-Atlantic Corals at Regional and Basin Scales. PLoS ONE 6(7): e22298.</p> <p>Figueira de Paula, A.; Creed, J. C. (2004). Two species of the coral <i>Tubastraea</i> (Cnidaria, Scleractinia) in Brazil: a case of accidental introduction. Bulletin of Marine Science. 74: 175-183.</p> <p>Cairns, S.D. A revision of a shallow-water azooxanthellate Scleractinia of the western Atlantic. Studies on the natural history of the Caribbean region, v. 75, p. 1-240, 2000.</p> <p>Mantelatto, M. C.; Creed, J. C.; Mourão, G. G.; Migotto, A. E.; Lindner, A. (2011). Range expansion of the invasive corals <i>Tubastraea coccinea</i> and <i>Tubastraea tagusensis</i> in the Southwest Atlantic. Coral Reefs. 30(2): 397-397.</p> <p>Fofonoff, P.W.; Ruiz, G.M.; Steves, B.; Carlton, J.T. (2014). National Exotic Marine and Estuarine Species Information System (NEMESIS)</p> <p>MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33).</p>

Group	Species	Native range	Known impacts	Source	Vectors / pathways	References
Cnidaria	<i>Tubastraea tagusensis</i>	East Pacific	competes with native organisms	MMA, 2009	Oil platform and hull fouling; aquaria (known as sunflower coral)	<p>Lages, B.G.; Fleury, B.G.; Pinto, A.; Paula, A.F. de; Creed, J.C. Chemical defenses against fish produced by two introduced scleractinian corals <i>Tubastraea coccinea</i> and <i>Tubastraea tagusensis</i> in Ilha Grande Bay - Brazil. In: CONGRESO LATINOAMERICANO DE CIENCIAS DEL MAR, 11., 2005, Viña del Mar, Chile. Resumo. p. 49-51.</p> <p>Paula, A.F. de; Creed, J.C. Two species of the coral <i>Tubastraea</i> (Cnidaria, Sclerectinia) in Brazil: a case of accidental introduction. Bulletin of Marine Science, v. 74, n. 1, p. 175-183, 2004.</p> <p>Creed, J.C. Two invasive alien azooxanthellate corals, <i>Tubastraea coccinea</i> and <i>Tubastraea tagusensis</i>, dominate the native zooxanthellate <i>Mussismilia hispida</i> in Brazil. Coral Reefs, v. 25, p. 350, 2006.</p> <p>MMA, 2009. Informe sobre as espécies exóticas invasoras marinhas no Brasil / Ministério do Meio Ambiente; Rubens M. Lopes/IO-USP... [et al.], Editor. – Brasília: MMA/SBF, 2009. 440 p. ; il. color. (Série Biodiversidade, 33)</p>

## **Annex 3 (chapter 6): Analysis of Similarities (ANOSIM routine from Primer E)**

One way analysis among the three different groups of salinity after one week of exposure

**(Global R): 0.564 (0.2%)**

	<b>R</b>	<b>Significance</b>
<b>Groups</b>	<b>Statistic</b>	<b>Level %</b>
<b>7, 20</b>	<b>0.748</b>	<b>0.8</b>
<b>7, control</b>	<b>0.956</b>	<b>0.8</b>
<b>20, control</b>	<b>-0.18</b>	<b>87.3</b>

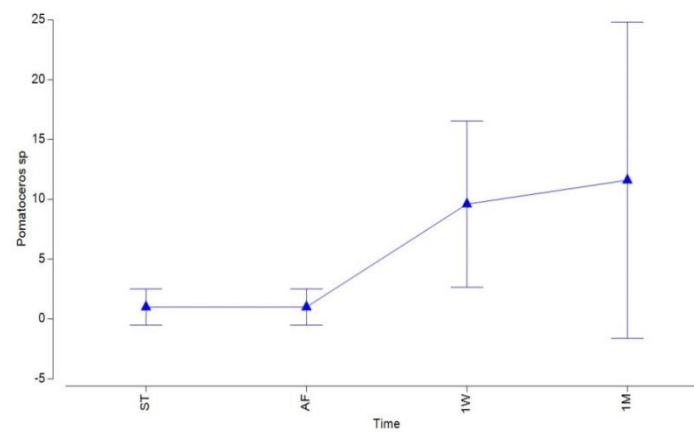
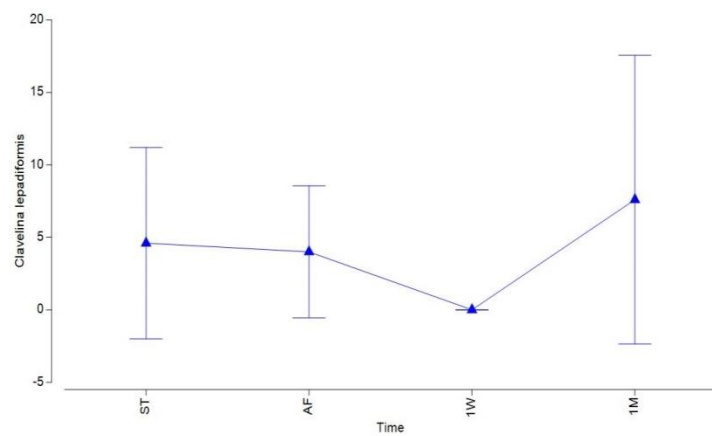
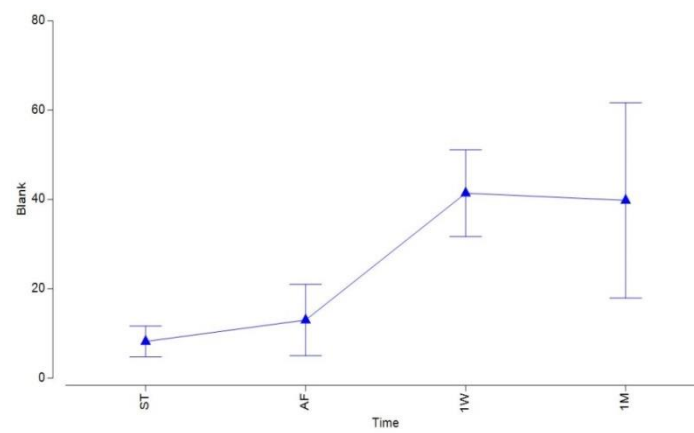
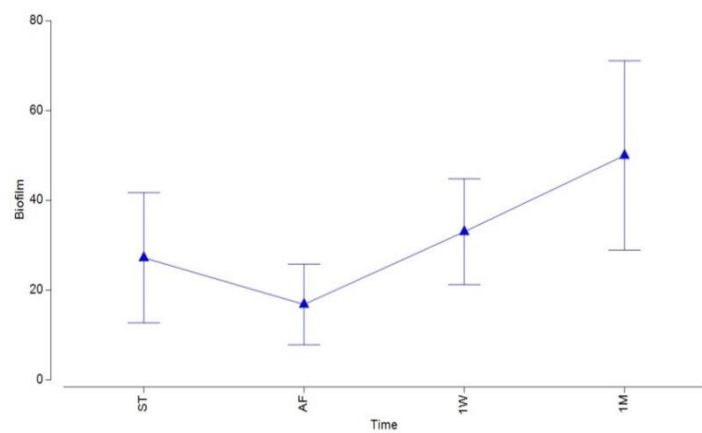
One way analysis among the three different groups of salinity after one month of exposure

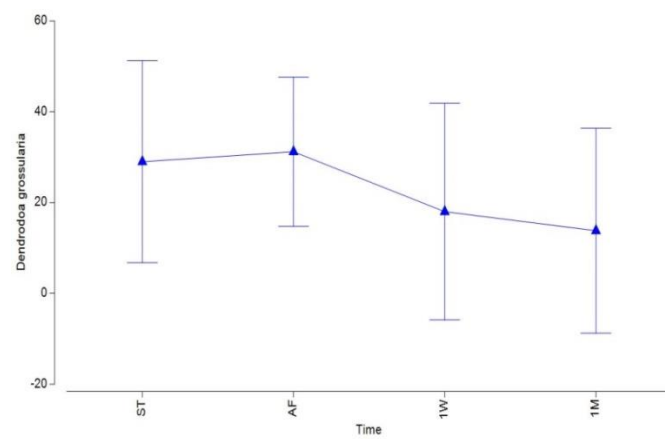
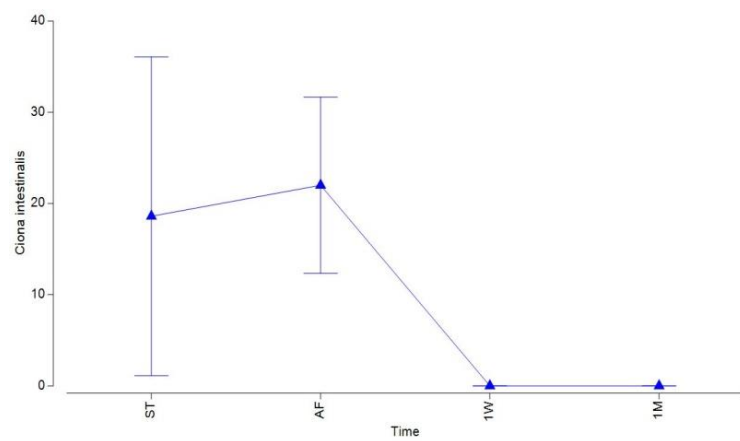
**(Global R): 0.518 (0.1%)**

	<b>R</b>	<b>Significance</b>
<b>Groups</b>	<b>Statistic</b>	<b>Level %</b>
<b>7, 20</b>	<b>0.764</b>	<b>0.8</b>
<b>7, control</b>	<b>0.744</b>	<b>0.8</b>
<b>20, control</b>	<b>-0.004</b>	<b>53.2</b>



**Annex 4 (chapter 6): 7 psu mean plots over time for biofilm, blank spaces, *Clavelina lepadiformis*, *Pomatoceros sp*, *Ciona intestinalis* and *Dendrodoa grossularia* (ST: before treatment, AF: immediately after treatment, 1W: one week after treatment and 1M: one month after treatment).**





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**Castro, M. C. T.**, Fileman, T. W., & Hall-Spencer, J. M. (2017). Invasive species in the Northeastern and Southwestern Atlantic Ocean: A review. *Marine pollution bulletin*, 116(1-2), 41-47.

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